Universidade de Lisboa

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Advances in the development of a stable transfection system for *Babesia ovis*

Catarina Maria da Silva Rosa

Supervisor: Sandra Isabel da Conceição Antunes, PhD, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa
Co-supervisor: Professor Madalena Maria Vilela Pimentel, Faculdade de Farmácia, Universidade de Lisboa

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Abstract

Babesia species, etiological agents of babesiosis, a recognized emerging vector-borne zoonose, are a significant animal and human health concern with a worldwide socio-economic impact. Genetic manipulation methods are pivotal to improve knowledge regarding the biology of these poorly studied parasites towards better disease control strategies. For *Babesia ovis*, responsible for ovine babesiosis, a tick-borne disease of small ruminants, these tools are not yet available.

Transfection technologies have a wide range of applications, being particularly useful in the study of the parasite-host cell interactions. The establishment of parasites expressing a fluorescent marker has allowed imaging of tick vector colonization, an approach that can also be applied to clarify *B. ovis* and related *Babesia* spp. life cycle events in the tick vector tissues. Thus, the main goal of this study was to set up basis for the development of a stable transfection system, driving expression of a fluorescent marker for *B. ovis*.

The present work has four objectives, (1) screening of regulatory regions in *B. ovis* genome and/or identification of heterologous promoters from other *Babesia* species, (2) development of transient transfection systems with the previously selected regions and evaluation of transfection conditions, promoter activity, transfection efficiency, (3) identification of a proper selection system for upcoming stable transfection of *B. ovis* and (4) the development of the plasmid construct for *B. ovis* stable transfection.

The study was based on the existence of interchangeable cross-species functional promoters between *Babesia* species. Therefore, first steps consisted in the development of transient transfection constructs with *elongation factor 1-alpha (ef-1α)* promoter regions from *B. bovis* and *B. ovata* to drive expression of the luciferase reporter gene. A promoterless plasmid was also developed to use as negative control in the luminescence assays.

Herein, we describe for the first time *B. ovis* transient transfection using heterologous promoters, the *ef-1α-B* intergenic regions from *B. bovis* and *B. ovata*. Their ability to drive expression of a reporter luciferase in *B. ovis* supports their cross-species functionality. The *ef-1α-B* promoter region from *B. ovata* resulted in the expression of high luciferase levels, thus an appropriate promoter for stable gene expression. Transfection efficiency was evaluated through qPCR to preclude the hypothesis that higher luminescence values were related with a higher transfection efficiency.

Based on available sequences from *B. bovis*, *B. bigemina* and *B. divergens*, PCR experiments were performed aiming the search for regulatory elements in *B. ovis* genome.

Analysis of the *actin* 5' flanking region revealed homology with *B. bovis actin* promoter but curiously the absence of a transcriptional start site consensus motif common between *B. bovis* and *Theileria* species. Regarding *ef-1α* and *rhoptry associated protein -1 (rap-1)* locus, their organization in *B. ovis* remains unclear but it was possible to retrieve a *rap-1* intergenic region with transcription termination signals, essential for the development of future autologous transfection systems. Also, a potential region for stable construct integration has been sequenced, the *ef-1α* gene.

Evaluation of *B. ovis* sensitivity to WR99210 and blasticidin-S, antibiotics commonly used to select apicomplexan transfectants, has been performed and blasticidin-S was identified as a selectable marker for future stably transfected *B. ovis*.

Currently, efforts are being conducted in the construction of a stable transfection plasmid with the *ef-1α-B* promoter region from *B. ovata* driving expression of a fluorescent reporter gene and an antibiotic resistance gene. The establishment of a *B. ovis* lineage expressing a fluorescent reporter gene will be applied to study *B. ovis* life cycle, enlightening interactions with the host cells.

Keywords: *Babesia ovis*, transient transfection, *elongation factor-1alpha*, plasmid construction, luciferase, sensitivity assays

Resumo

Os parasitas do género *Babesia* são agentes etiológicos de babesiose, uma zoonose emergente transmitida por carraças, reconhecida com uma ameaça importante para a saúde animal e humana e com um impacto socioeconómico a nível mundial. Métodos de manipulação genética são essenciais para o conhecimento da biologia destes pouco estudados organismos, de forma a melhorar as estratégias para o seu controlo. Em *Babesia ovis*, agente responsável pela babesiose ovina, tais metodologias não foram, até à data, desenvolvidas.

A transfecção é uma metodologia que têm inúmeras aplicações, sendo particularmente útil no estudo de interações parasita-célula hospedeira. O estabelecimento de uma linhagem de parasitas a expressar um marcador fluorescente permitiu monitorizar a colonização do vector pelo agente patogénico. Esta estratégia pode ser também aplicada para clarificar eventos do ciclo de vida de *B. ovis* e de outras espécies do género *Babesia*, na carraça vector. Desta forma, o principal objetivo do presente estudo foi o de criar os pilares necessários ao desenvolvimento de um sistema para transfecção estável capaz de expressar um marcador fluorescente em *B. ovis*.

O presente trabalho apresenta quatro objetivos, (1) o *screening* de regiões reguladoras no genoma de *B. ovis* e/ou a identificação de promotores heterólogos noutras espécies do género *Babesia*, (2) o desenvolvimento de sistemas para transfecção transiente com as regiões reguladoras previamente selecionadas bem como a avaliação de parâmetros da transfecção, atividade das regiões promotoras e eficiência da transfecção, (3) identificação de um sistema de seleção apropriado para isolar *B. ovis* transfectada de forma estável e (4) o desenvolvimento de um plasmídeo para transfecção estável de *B. ovis*.

Este estudo baseou-se na existência de promotores funcionais e intercambiáveis entre espécies do género *Babesia*. Sendo assim, o trabalho iniciou com o desenvolvimento de plasmídeos para transfecção transiente contendo uma das regiões promotoras do *factor de elongação-1alfa* (*ef-1α*) de *B. bovis* e *B. ovata* permitindo a expressão do gene repórter que codifica para a luciferase.

O presente trabalho descreve, pela primeira vez, a transfecção transiente de *B. ovis*, sendo que ambas as regiões promotoras utilizadas permitiram a expressão da luciferase em *B. ovis* corroborando a sua funcionalidade entre espécies. Em particular a região promotora de *B. ovata* induziu a expressão de níveis elevados de luciferase, revelando ser uma região promotora adequada para incorporar num sistema que permita uma transfecção estável. A eficiência de

transfecção foi avaliada através de análise por qPCR de forma a validar que as diferenças observadas nos valores de luminescência não estavam relacionadas com diferenças na eficiência do processo de transfecção.

Tendo por base sequências de *B. bovis*, *B. bigemina* e *B. divergens*, foram realizados PCR com objetivo de amplificar regiões reguladoras no genoma de *B. ovis*. Análise da região 5' flanqueante da *actina* revelou homologia com a região promotora da *actina* em *B. bovis*, embora, curiosamente, a sequência consenso em *B. bovis* e espécies do género *Theileria* para iniciação da transcrição estivesse ausente. Relativamente ao locus do *ef-1α* e da *rhoptry associated protein -1 (rap-1)*, a sua organização ainda não está esclarecida tendo sido possível obter a região intergénica de *rap-1*. Esta região apresenta sinais de terminação podendo ser utilizada para um futuro sistema para transfecção constituído por regiões reguladoras autólogas. Foi ainda sequenciada uma possível região para integração de um construto estável no genoma de *B. ovis*, o gene *ef-1α.*

A blasticidina-S foi selecionada como agente selector de *B.ovis* transfectados após ensaios de suscetibilidade aos antibióticos WR99210 e blasticidina-S, comummente utilizados para selecionar espécies transfectada do filo apicomplexa.

O desenvolvimento de um plasmídeo para transfecção estável está em curso o que permitirá estabelecer uma linhagem de *B. ovis* a expressar a proteína fluorescente vermelha (RFP). Os resultados do presente trabalho decerto irão contribuir para melhor compreender o parasita bem como interações com células hospedeiras.

Palavras-chave: *Babesia ovis*, transfecção transiente, *factor de elongação-1alpha*, construção de plasmídeos, luciferase, ensaios de suscetibilidade

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Abbreviations

5' act	5' flanking region of *actin* gene
BLAST	Basic Local Alignment Search Tool
*Bo*SPD	*Babesia ovis surface protein D* gene
Bp	Base pair
Bsd	*blasticidin deaminase* coding gene
CMV	Cytomegalovirus
DMSO	Dimethyl sulfoxide
hdhfr	*human dihydrofolate reductase* coding gene
ef-1α	*elongation factor 1-alpha* gene
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
IC_{50}	50 % inhibitory concentration
IG	Intergenic
iRBC	Infected red blood cells
IRES	Internal ribosome entry sites
LB	Luria-Bertani
Luc	*firefly luciferase* coding gene
TES	*N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid
ORF	Open reading frame
PAS	Polyadenylation signal
PCR	Polymerase chain reaction
Pol II	RNA Polymerase II
PPE	Percentage of parasitized erythrocytes
qPCR	Quantitative real-time PCR
rap-1	*rhoptry associated protein-1* gene
RFP	Red Fluorescent Protein
RLU	Relative luciferase unit
rRNA	Ribosomal RNA
SD	Standard deviation
s.l.	Sensu lato
S.O.C.	Super Optimal broth with Catabolite repression

spp.	Species
s.s.	Sensu stricto
SV40	Simian virus 40
TSS	Transcription Start Site
UTR	Untranslated region
VESA	Variant erythrocyte surface antigens

1. Introduction

Development of genetic manipulation methods for *Babesia ovis* can provide an essential breakthrough for the study of this parasite basic biology, including its life cycle dynamics, parasite-vector and parasite-host interactions, ultimately enabling rational design of more effective drugs and vaccines towards disease control [1,2].

Currently, scarce information regarding intra-tick *Babesia* species (spp.) dynamics exists, even though targeting key developmental stages in tick tissues presents as an attractive way towards the development of transmission-blocking vaccines. Such approach demands a deep knowledge of conserved and divergent developmental features between *Babesia* spp. [3].

Transfection technology (incorporation and expression of foreign DNA or RNA) has a wide range of applications, particularly useful for the study of parasite-host cell interactions. Recently, it was possible to image pathogen colonization in its tick vector by transforming the bacteria *Borrelia* sp. to stably express a fluorescent marker, thus the same strategy could be applied to understand *B. ovis* and related *Babesia* spp. life cycle events in the tick vector tissues [4]. Even though there is very limited knowledge regarding *B. ovis-Rhipicephalus bursa* interactome, some candidate protective antigens have been identified recently in the tick vector salivary glands, as having an important role in biological functions, such as tick development, feeding and pathogen transmission [5]. Transfection methods stand out also in the vaccinomics research currently ongoing for the *B. ovis-R. bursa* interface, by enabling targeted disruption of genes of interest leading to the identification of key/essential parasite gene products for successful transmission [2,5–8]. Moreover, the same gene manipulation strategy can be applied to the study of virulence factors, particularly those that interact with effectors produced by the host immune system. The phenotypic characterization of mutants lacking or with partially functional virulence factors can be a powerful method to characterize these factors, which can be potential targets for vaccine development [2,9]. Other possible endpoint of this tool is the design of novel-life transfection-based vaccines, in which the host is vaccinated with attenuated-live vector vaccine containing a stably transfected tick antigen, eliciting the host protective immune response against both the parasite and the tick [10,11].

Babesia spp. have many characteristic features that make this parasite especially suitable for the development of genetic manipulation methods and posterior identification of drug targets [12]. A small genome size in comparison to other apicomplexan parasites simplifies comparative genomic studies and, the relatively reduced number of genes concomitantly with being haploid in the asexual cycle also facilitates the discovery of drug targets [13–15]. Moreover,

it appears that the integration of exogenous DNA in *Babesia* spp. genome occurs exclusively by a homologous recombination mechanism which supports the development of gene knockout experiments for functional studies [8]. Currents challenges for the development of such tools are related with scarce of appropriately annotated genomes and, particularly for *B. ovis*, the unavailability of its genome sequence in public databases [13,15–18].

The "One Health" concept recognizes that Human, animal and environment Health are tightly intertwined, demanding synergistic interdisciplinary collaborations to, among other purposes, pave the way for the expansion of scientific knowledge. Research projects that aim the control of tick and tick-borne diseases have been positioned in the framework provided by this initiative, prioritizing the use of "omnics" and systems biology approaches concomitantly with the development of genetic manipulation methods [2,19].

1.1. *Babesia* and Babesiosis

The genus *Babesia* (phylum Apicomplexa, order Piroplasmida) comprises protozoan hemoparasites responsible for babesiosis, a recognized emerging vector-borne zoonose. More than one-hundred *Babesia* spp. have been identified so far, remarkably able to establish infections in a wide variety of domestic and wild animals, with some species having the ability to infect humans. These organisms are considered the second most prevalent blood-borne parasites of mammals after trypanosomes and, giving the worldwide distribution of their tick vectors, babesiosis is recognized as the most common blood disease of free living animals [20–25].

Babesia spp. were named after Victor Babes, the bacteriologist that provided their first description in 1888, after observation of these microorganisms inside erythrocytes of cattle presenting hemoglobinuria and later in sheep blood. In 1893, Smith and Kilbourne identified this intraerythrocytic parasite as the etiological cause of Texas Cattle Fever and also the role of ticks as vectors, being the firsts to demonstrate that an arthropod was the carrier of a disease agent [26–28]. Indeed, transmission usually occurs through a *Babesia*-infected tick bite but it can also happen via blood transfusion or congenital acquisition during pregnancy [27].

Babesiosis is acknowledged for having a great impact at economic and social levels, particularly in developing countries. In addition, the intensification of human and animal movements combined with environmental changes and the geographical expansion of several tick species is contributing to the rise of this tick-borne disease as a global threat [29–31].

From the veterinary point of view, bovine babesiosis, caused by *Babesia bovis* and *Babesia bigemina*, has drawn more attention due to the economic burden imposed, linked to

mortalities, abortions, decreased milk and meat production, control measure costs, losses in potential production and cattle trade restrictions [21,32,33]. Despite existence of tick vector eradication programs disease outbreaks occur often and it is considered that most of the 1.2 billion cattle worldwide is exposed to babesiosis [34–36]. Moreover, ovine babesiosis caused mainly by *B. ovis* has a major economic impact and companion animals, such as dogs, are also severely affected with infections caused by *Babesia vogeli* and *Babesia gibsoni* [21,37–40].

From the medical point of view, human babesiosis, caused by an increasing diverse range of species, mainly attributed to *Babesia microti* and *Babesia divergens*, is still not recognized as a neglected disease [27,41–45]. Even though humans are only considered accidental hosts for *Babesia* spp., there is a global concern regarding this emerging zoonosis since human babesiosis incidence is increasing with several clinical cases being reported recently from many countries worldwide, as reviewed by Yabsley and Shock., 2013 and Ord and Lobo, 2015 [27,43]. Moreover, babesial infections are often overlooked due to the relative unspecific clinical signs and lack of medical awareness for this disease, with underreporting of human cases being a major issue [45,46].

Babesiosis clinical signs arise when the rate of erythrocyte (infected) loss surpasses the rate of their replacement, giving rise to anemia and its associated health issues. Therefore, babesial infections progress with quite different degrees of severity depending of hosts' age, immunological status, co-infections with other pathogens, and/or genetic factors. Acute babesia infections manifestations include, besides anemia, fever, hemoglobinuria, jaundice, malaise, lethargy and anorexia while the chronic status is usually asymptomatic. Nonetheless, these surviving hosts frequently become carriers and maintain ticks infection cycle [21]. *Babesia* spp. are responsible for diseases of veterinary and medical importance through transmission by their different tick vectors **(Table1)**.

Table 1. Distribution of several *Babesia* spp., their main tick vectors and hosts.

***Babesia* spp.**	**Main vectors**	**Main hosts**	**Distribution**	**Ref**
B. bovis	*Rhipicephalus microplus / Rhipicephalus annulatus*	Cattle (*Bos taurus*)	Africa, America, Asia, Australia, Europe	21
B. bigemina				
B. ovata	*Haemaphysalis longicornis*		Asia	47
B. divergens *	*Ixodes ricinus*		Europe and Asia	48,49
B. ovis	*Rhipicephalus bursa*	Sheep (*Ovis aries*) and goat (*Capra aegagrus*)	Africa, Asia, Europe	50
B. gibsoni	*Rhipicephalus sanguineus*	Dog (*Canis lupus*)	Africa, America, Asia, Australia, Europe	39,51,52
B. vogelli				
B. microti *	*Ixodes scapularis*	White-footed mouse (*Peromyscus leucopus*)	America, Europe and Asia	53–55

*Human are accidental hosts for these *Babesia* species. **Adapted from:** Schnittger *et al.*, 2012 [21].

1.1.1. *Babesia* life cycle

Babesia life cycle is maintained in a complex system of tick vectors and vertebrate reservoirs (**Figure 1**). A generic *Babesia* life cycle is currently accepted, even though there is the major controversy regarding variants across the genus, as reviewed by Jalovecka *et al.*, 2018 [3]. In the vertebrate hosts these parasites reproduce asexually within the erythrocytes (merogony) and are referred as piroplasms or piroplasmids due to their pear-shaped appearance, sharing the term with *Theileria*, a phylogenetically related protozoan. The initial *Babesia* sexual stages, referred as gametocytes, appear with successful transmission to its vector, reaching the tick midgut together with the blood meal, differentiating into gametes. After fertilization these give rise to a zygote that undergoes a meiotic division to originate kinetes (gamogony) and a final cycle of asexual multiplication originates sporozoites (sporogony), the host-invasive stage, guarantying a successful infection of the host. In the tick vector occurs transstadial transmission, between the tick stages of development and, for most *Babesia* spp. transovarial transmission is present, a process mediated by parasite invasion of the ovarian cells and transmission via larval progeny to tick larvae. *Babesia* sensu stricto (s.s.) is the informal classification for the organisms that exploit this transovarial transmission strategy while *Babesia* sensu lato (s.l.) refers to species that are morphologically similar to *Babesia* but have different life cycle features or cannot be designated either *Babesia* or *Theileria* with certainty. Nonetheless, this transovarial transmission is recognized as a unique feature among all the apicomplexan parasites [3,14,21,25,43,56–59].

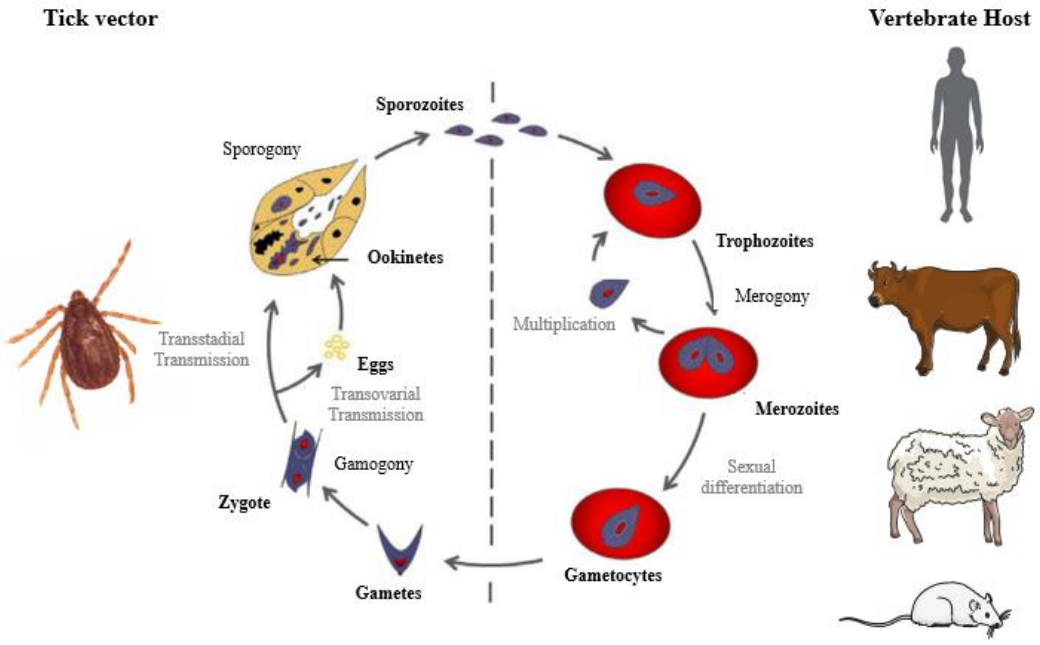

Figure 1. *Babesia* spp. generic life cycle. A susceptible tick, upon its blood meal, ingests these parasites, gametocytes, from an infected host. In the tick midgut, they undergo sexual development into mature gametocytes

and gametes, whose fusion results in zygotes, able to invade midgut cells. These zygotes go through meiosis and differentiate into motile prolonged kinetes, ookinetes, (gamogony) that escape midgut cells and spread to different tissues throughout the haemolymph, including the salivary glands. Here, kinetes undergo a cycle of asexual multiplication originating sporozoites (sporogony) that will infect a naïve vertebrate host during feeding. The infection is maintained throughout tick developmental stages (transstadial transmission). For *Babesia* sensu stricto, kinetes invade tick ovaries and eggs resulting in infected larvae (transovarial transmission). In the vertebrate host, upon erythrocytes invasion, parasites reproduce asexually (merogony). [14,21,25]. **Adapted from**: Schnittger *et al.*, 2012 [21].

1.1.2. *Babesia* evolution and comparative genomics: applications

Molecular phylogeny data contributes to more robust *Babesia* spp. taxonomic classifications, establishment of co-evolution patterns with its tick vectors and vertebrate hosts, enlightening evolutionary lineages [21,60]. Phylogenetic studies using the *18S ribosomal RNA* (*rRNA*) gene separates *Babesia* spp into six broad clades with different degrees of support with the clear distinction between two major lineages: *Babesia* s.s. and *Babesia* s.l. [21,61]. Based on this *18S rRNA* sequence, *B. ovis* is placed in *Babesia* sensu stricto closely related to *B. bovis,* assembling with *B. orientalis* in a group of moderate support, separated from the branch comprising *B. bigemina*, *Babesia ovata*, *Babesia major*, *Babesia crassa* and *Babesia motassi.* Nonetheless, all of these species are placed in the same phylogenetic clade, clade VI. In a more recent study using C1A cysteine proteinases sequences this hypothesis was further supported. Also, it has been considered that, within Piroplasmida, the most rapidly evolving lineages are those in the clade with *B. bovis* and *B. ovis* [21,62–64].

As an alternative to analyzing *18S rRNA* sequences alone, mitochondrial genome sequences and structures have proven to be useful for elucidation of evolutionary relationships and recently with was possible to identify five distinct *Piroplasmida* lineages with Babesia *spp.* being assigned to three different groups, referred as the *Babesia microti* group, the Western *Babesia* group, and *Babesia* s.s..[59].

Babesia bovis genome was the first to be reported with a total size of 8.6 mega base pairs (Mbp) [13]. Since then other *Babesia* species genomes have been sequenced, *B. divergens* (10.8 Mbp), *B. bigemina (*13.8 Mbp), *B. microti* (6.5 Mbp), *Babesia sp Xinjiang* (8.4 Mbp), *Babesia orientalis* and *B. ovata* (14.4 Mbp) [15,17,18,65,66]. *B. ovis* genome has also been sequenced however it is not publicly available [62] . For *B. bovis* it was found that genome organization and structure was remarkably similar to *Theileria*, presenting microregional similarity to *Plasmodium falciparum* despite the similarities with the last concerning the infection clinical outcomes.

Exploring evolutionary lineages alongside with genome sequencing provides a potential avenue to the development of a pan-babesiacidal or even a more wide-range therapeutic

strategy. Genomic comparative analyses between *T. parva* and *B. bovis* has allowed to identify potential antigens in *B. bovis* that were already under study as vaccine candidates for theileriose [13,67]. Particularly, in *B. ovis*, a simple gene comparative analysis using C1A family cysteine protease sequences from *B. bovis*, resulted in the identification of papain-like cysteine protease, ovipain-2, an attractive potential vaccine target [68].

Using the same approach, comparing *B. ovata* to close related apicomplexan, such as *B. bigemina*, *B. bovis*, *B. microti*, *P. falciparum* and *Toxoplasma gondii*, allowed to conclude that the limited diversity of the *ves* genes, which encode variant erythrocyte surface antigens (VESAs) related with the parasite ability to evade or suppress host immune responses, could explain the lower virulence of this species [18]. Likewise, this multi-gene family *ves* in *Babesia* sp. Xinjiang, a species known for having a limited virulence/pathogenicity in sheep was found to be substantially distinct both in number and sequence from those of *B. bovis*, *B. bigemina* and *B. divergens* [17].

In sum, genomic comparison facilitates the identification of genes that are undergoing positive selection and gene family expansions or contractions providing insights into the evolution of gene families and their possible roles in virulence and pathogenicity, which can be improved by using model apicomplexan parasites for functional studies [17]. The ability to genetically manipulate *Babesia* spp. genome, namely *B. ovis*, a sheep and goat-infecting pathogen phylogenetically close to *B. bovis* and recognized for having a high virulence for its hosts, could be a platform for understanding the role of key molecules in host invasion and intra-tick development.

1.2 *Babesia ovis*

Babesia ovis is the main etiological agent of ovine babesiosis, a tick-borne disease of small ruminants prevalent in Eastern Asia, Southern Europe (Mediterranean basin), the Middle East and Northern Africa, a geographical distribution overlapping the one of its main vector, the tick *R. bursa* [38,69]. Ovine babesiosis is an acute disease whose onset is characterized by high fever, a scenario that progresses to other clinical symptoms such as hemolytic anemia, hemoglobinuria, icterus and, in more severe cases, pancytopenia occurs. The untreated cases usually lead to death and even upon treatment the animal may die as a result of a heavy infection or suffer a disease relapse after the therapy period [70]. Recently, an outbreak has occurred in Spain, demonstrating the deleterious effect of *B. ovis* in naïve sheep transferred from a tick-free region to a *R. bursa*-infested region with endemic piroplasmosis [71].

Ovine babesiosis is acknowledged for having an important economic impact related with animal mortality, productivity losses (decrease of milk, meat and wool production) and costs of the livestock treatment. Particularly for underdeveloped countries, social impact is quite significant in the livehood of resource-poor farmers and in food security[72,73]. The major disease control method consists in the use of a babesiacide, imidocarb dipropionate, to manage the clinical signs. Even though its efficacy has been long-established, safety issues have emerged related with milk contamination and potential carcinogenicity of the drug [74–76]. The control of the tick vector infestations is another approach to manage this tick-borne disease however, emergence of acaricide resistance, including in *R. bursa* species, exposes the vulnerability of this strategy [77]. The need for safer and more effective strategies is clear and for areas with a *B. ovis* unstable endemic status, the requirement of an immunization programme has been established [77,78]. Some efforts were conducted in the context of the development of a live attenuated vaccine but the inability to eliminate *B. ovis* virulence after several blood passages invalidates, to date, this therapeutic approach [79]. Alternatively, the research paradigm can be focused in the development of subunit vaccines that target the reduction of tick infestations and block pathogen transmission [80–82]. Insight on the molecular interactions in the tick-pathogen interplay and the consequent identification of suitable antigenic targets are presently key to implement this strategy [83]. Research focusing *Babesia* spp., as well as other apicomplexan parasites, encompasses significant constraints such as maintenance of *in vitro* parasite cultures, unavailability of complete and well annotated genomes and practical tools for their genetic manipulation [2]. Regarding the first, *in vitro B. ovis* Israeli strain cultures have been successfully established (**Figure 2**), however genome sequencing availability and methods for genetic manipulation remain a necessity [84].

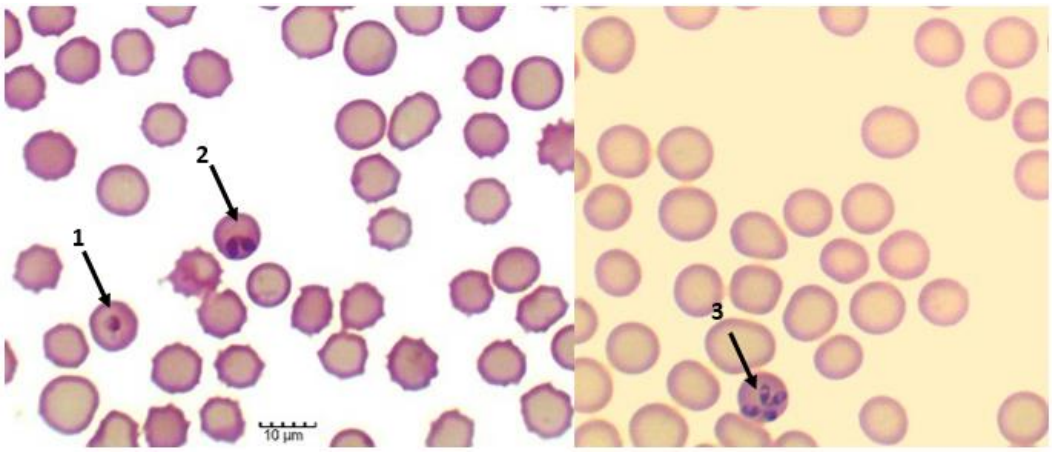

Figure 2. *Babesia ovis* forms inside lamb erythrocytes in in vitro cultures. 1. Single round trophozoite. **2**. Merozoite characterized by piriform shapes forming a pair. **3.** Merozoite characterized by piriform shapes

forming a tetrad ("Maltese cross" pattern). Intraerythrocytic parasites were observed under a 400x original magnification of a Motic BA210 LED trinocular compound microscope (original from the author).

1.3 Genetic manipulation Methods for *Babesia* spp.

Transfection is the process of introducing exogenous nucleic acid molecules (DNA or RNA) into eukaryotic cells. The transferred nucleic acid might reside in the cell either stably or transiently depending of the nature and destination of the genetic material (**Figure 3**). Briefly, transient transfection techniques are designed to introduce exogenous DNA and short-term expression of the foreign genetic material. Thus, the introduced nucleic acid does not integrate into the genome of the target cells, and the transfected DNA will not be replicated. On the other hand, in stable transfection, the introduced genetic material, that usually has a marker gene for selection (transgene) integrates the host genome and maintains the transgene expression along host cells replication [85].

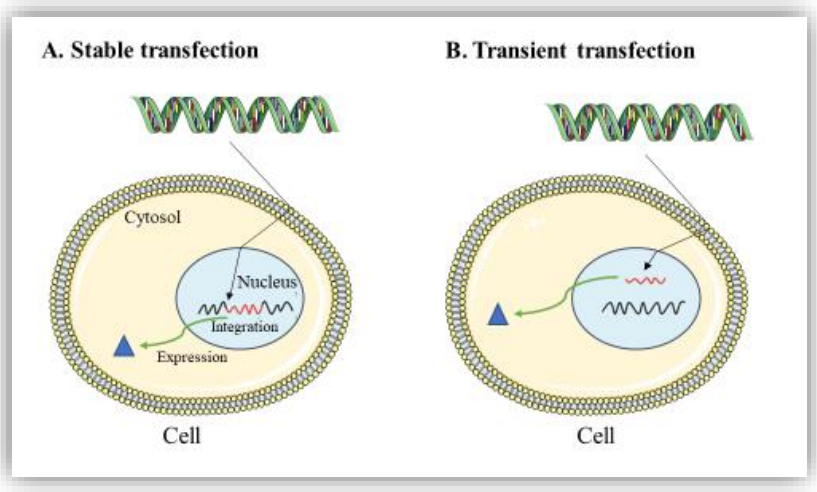

Figure 3. Schematic diagrams of two different transfections. (A) Stable transfection. Foreign DNA (red wave) is delivered to the nucleus by passage through the cell and nuclear membranes. Foreign DNA is integrated into the host genome (black wave) and expressed sustainably. **(B).** Transient transfection. Foreign DNA is delivered into the nucleus but is not integrated into the genome. Triangles are expressed proteins from transfected nucleic acids. Black arrows indicate delivery of foreign nucleic acids. **Adapted from:** Kim *et al.*, 2010 [85].

Transfection systems have been developed for several apicomplexan parasites with the initial report of the successful application of transfection technology described in *T. gondii* [86]. Since then, transient and stable transfection systems were also developed for some *Plasmodium* spp. and for *Neospora caninum* a transient transfection was promptly developed based on the work done with *T. gondii* [87–89]. For *Babesia* spp. such manipulation emerged in the last decade after availability of *B. bovis* genome sequence with reports of transient transfections system for this specie [136,90–92]. A stable transfection system for *B. bovis* was developed and it is currently acknowledged that for at least *T. gondii* and *B. bovis* a stable transfection baseline

protocol was defined [2,93]. More recently, transient transfection systems followed by stable transfection were performed in *B. ovata* after the unravelling of its genome [18,94]. Similarly, stable transfection systems have been developed for *B. bigemina* and *B. gibsoni* [95–98] (**Figure 4**).

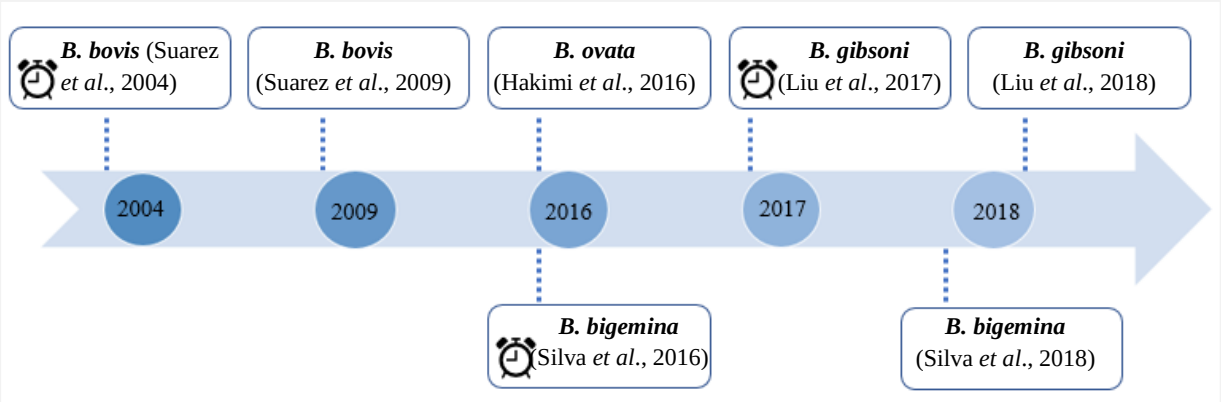

Figure 4. Timeline of selected landmark publications on the development of transient and stable transfection systems in *Babesia* spp. ⏰ indicates transient transfection system.

Transient transfection systems establishes the appropriate parameters for introduction of the exogenous DNA (definition of an electroporation/nucleofection protocol that carries the minor impact for the parasite) and, more importantly, permit to identify and test function and efficacy of promoter and termination signals (regulatory elements) mediating gene expression and regulation [6,99]. In fact, these systems were essential to find suitable promoters in *Babesia* spp. and to reveal the existence of promoters with interchangeable cross-species function [6,94,95,97,100]. The establishment of a stable transfection system demands, besides the aforementioned criteria, to identify a suitable genetic marker to select the transgenic parasites (a selectable marker) and the preparation of a cassette containing the defined selectable marker gene along with suitable targets for DNA integration, whose disruption does not compromise the parasite viability [6,2] . Therefore, to establish a stable transfection system for *B. ovis* that enables the expression of a fluorescent reporter protein, the methodology followed should be similar to the ones used previously for transgenic- *Babesia* spp., following the steps described in **figure 5.**

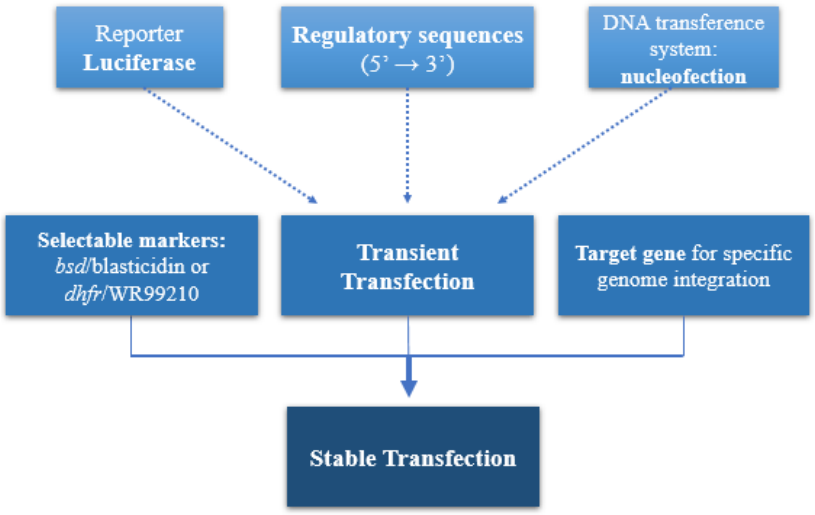

Figure 5. Flowchart describing the steps required for the development of a stable transfection system for *Babesia ovis*. Adapted from: Suarez *et al.*, 2010 [6].

1.3.1. DNA transfer system: nucleofection

There is a huge methodological diversity concerning transfection, with the several strategies being broadly classified as biologically, chemically, and physically mediated methods [85]. Herein, focus will be on a physical method, electroporation, since it is regarded as the most robust and reproducible method to transfect Apicomplexan parasites [101].

Electroporation is the most commonly used physical method of DNA delivery and its exact mechanism is unknown. However, it is suggested that a short electrical pulse disturbs cell membranes, creating holes through which nucleic acids can pass. It is accepted that it occurs through a multistep mechanism that relies not only on the plasma membrane permeabilization but also in the electrophoretically driven interaction between the DNA molecule and the destabilized membrane, both mediating its passage across the membrane and, once inside the cell, its migration towards the nucleus [85,102].

Babesia merozoites are obligatory intraerythrocytic organisms thus, a successful transfection approach must be able to transfer the exogenous DNA through the erythrocyte, merozoite and nuclear membrane layers. This results in a severe impairment in DNA delivery as seen in the low transfection efficiencies for this parasite and related apicomplexan [92,103,104]. For *B. bovis* the process of developing a transient transfection system involved the determination of suitable electroporation parameters and later a comparison between that process and nucleofection, a novel electroporation based method [92,99]. Nucleofection consists in a transfection method that combines a specific nucleofector solution and specific electrical parameters to successfully deliver DNA into the nucleus. It is though that during nucleofection

a square wave pulse is produced which improves pore-formation and enables the incorporation of exogenous DNA in the nucleus without cell division [101,105].

Nucleofection has resulted in higher transfection efficiencies and enhanced parasite viability in *Plasmodium* [106,107]. For *B. bovis* it was shown that nucleofection was more efficient for the delivery of smaller plasmid DNA amounts and the inverse occurred with electroporation in which efficiency would increase concomitantly with the amount of DNA to deliver (to a maximum plateau of 200 μg), whereas delivery of 20 μg of DNA corresponded in both methods to similar relative efficiencies. Regarding the detrimental effects of any of the methods for the parasite it was shown that nucleofection provided a superior parasite viability [6,99,104].

Electroporation and nucleofection have been used for transfection of several *Babesia* spp. but an optimization of the electroporation parameters for the first and the assessment of the best nucleofector solution for the latter needs to be performed. For *B. bovis* it was suggested that nucleofector program v-024 in combination with "Plasmodium" buffer was optimal and for *B. ovata* the same program in conjunction with Amaxa nucleofector human T-cell solution was used to stably transfect the parasite in a Nucleofector™ 2b Device, an association also used to stably transfect *B. bovis* [7,92,94,108]. On the other hand, *B. gibsoni* was stably transfected using an optimized protocol consisting of Lonza buffer SF associated program FA113 of Amaxa 4D Nucleofector™ device [98,100]. For *B. bovis*, optimized electroporation settings (1.2 kV/ 25 μF/200 Ω) have been used successive times to transfect this parasite and the same protocol was used to stably transfect *B. bigemina* [6,10,93,96].

1.3.2. Regulatory sequences

1.3.2.1. Promoter regions and transcription start sites

The identification of transcription start sites (TSSs) is crucial for *in-silico* gene discovery, to understand transcription regulation mechanisms and to aid in the definition of the core promoter[109–111]. The latter, allied to the identification of *cis*-regulatory elements, binding sites for proteins involved in transcription initiation and regulation, it is essential to define the size of the isolated promoter for cloning. This information is key for the development of more efficient systems for transient and stable transfection [112,113]. Nonetheless, it must be taken in account that gene expression can also be regulated at post-transcriptional and translational levels, but little is known about this modulation in apicomplexan parasites. In *P. falciparum* the correlation between mRNA and protein expression is found moderately positive even though

there is some delay in some genes expression indicating the existence of such regulatory mechanisms [114,115].

It is known that for every eukaryotic gene there is a core promoter region in the 5′ untranslated region (UTR) containing ate least one TSS signal to which binds the RNA Polymerase 2 (Pol-II). Particularly, the 5′ UTRs between TSSs and the first in-frame initiation codons are recognized for modulating RNA stability through action of internal ribosome entry sites (IRESs) or riboswitches. This region, for *B. bovis,* has a median length of 152 bp, similar in size with the one from *T. gondii* (130 bp) [113,116–118]. This gene-proximal region is crucial for a nominal gene expression and additional upstream sequences enhance the promoter activity [119].

In *B. bovis* a TSS initiator-like motif TYAYWWW has been identified, probably a binding site for the general transcription factors TAF1 an TAF2, and also other location-specific consensus motifs, *cis* elements, have been found, probably crucial for binding to other transcriptional factors [113].

The apicomplexan parasites transcriptional machinery is recognized for differing from other eukaryotes, in which the absence of canonical eukaryotic transcription factors is particularly noticeable [113,120–124]. Additionally, it is acknowledged that apicomplexan transcription machinery is not able to recognize viral promoters, such as the cytomegalovirus (CMV) and simian virus 40 (SV40) that are widely used to produce heterologous transfection systems [125,126].

Herein, the most used promoter regions in *Babesia* spp. to drive expression of exogenous genes: the **5' flanking region** of the ***actin*** gene (**5' *act***) and the ***elongation factor-1 alpha* intergenic region** (***ef-1α* IG**) will be focused. The **5' *act*** has been used to drive expression of the selective marker cassette in stably transfected *B. bovis* and *B. ovata* [7,94]. More recently it was shown that 5' *act* from *B. gibsoni* presents higher activity in *B. bovis* than its homologous promoter and this promoter region was interchangeably functional between *B. bovis* and *B. gibsoni*[127]. The ***ef-1α* IG** contains a recognized strong constitutive promoter signal [91,128]. In *B. bovis* and *B. bigemina* the *ef-1α* locus consists in two identical *ef-1α* genes arranged in a head to head orientation separated by a 1.4 kb IG, structurally similar to the locus found in *Plasmodium* genome. Apparently, the IG contains two independent promoters (*ef-1α* IG-A and *ef-1α* IG-B) regulating bidirectional transcription of the two *ef-1α* open reading frames (ORFs), a bidirectional activity first described in *P. berghei* [91,95,129,130] Therefore, a rational approach for the identification of *B. ovis ef-1α* promoters consists in searching for this locus in its genome. The most active promoter region within the *ef-1α* IG region consists in a 710 bp region located

upstream the *ef-1a* gene B, the *ef-1α* IG-B [6,91]. This promoter region has been used to drive stable expression of a green and a red fluorescent-blasticidin deaminase-S (GFP-BSD and RFP-BSD) fusion proteins in *B. bovis* merozoites [93] and GFP expression in *B. ovata* and *B. gibsoni* [94,98]. Remarkably, the bidirectional transcription activity was used to express simultaneously a GFP-BSD and a tick antigen, being this the first experience of expression of a foreign antigen in a transfected *B. bovis* delivery platform, thus a live-vector vaccine [10,128,108].

Recently, it was found that promoters previously identified in *B. bovis ef-1α* IG region are also functional in *B. bigemina* and ten interchangeable cross-species functional promoters, including *ef-1α* IG-B, were identified between *B. gibsoni* and *B. bovis* [100]. Interestingly, the *ef-1α* IG heterologous promoter sequence showed to be more efficient driving expression of the reporter luciferase than the homologous promoter in *B. bigemina*, and similar results were found in the latter study with *B. gibsoni* 5' *act* being more active than the homologous promoter in *B. bovis* [95,100]. Finally, a study in *B. bovis* suggests that the use of heterologous regulatory regions such as cross-species functional promoters increases the specificity of the genome integration event [8]. Since the *B. ovis* genome is currently unavailable, these findings together with the availability of other genome sequences from *Babesia* spp., particularly the one of its phylogenetically close *B. bovis*, provides a solid working basis for the present study [16,65,77, 95,100].

1.3.2.1. Transcription termination regions

Transcription termination in eukaryotes consists in the release of the Pol II together with the nascent RNA from the DNA template, however the underlying molecular mechanisms are still under study [120,131,132]. Transcription termination is assumed to occur the same way as in the yeast system model, with the Pol II continuing transcription after the polyadenylation signal (PAS). Then, a succession of allosteric protein-protein interaction end in the transcript cleavage 11-30 bp downstream the PAS [120,132]. Around the transcription termination site, it has been observed, for the apicomplexan *T. parva*, the presence of over-represented motifs, some of them with the potential to produce a stem-loop structure (hairpin), possibly having a structural role in transcription termination [120,133].

The intergenic regions in the **rhoptry associated protein-1 *(rap-1*) locus** were used for the first transient transfection system in *B. bovis* promoting exogenous gene expression [90]. However, it was demonstrated that *rap-1* promoter signals were relatively weak and potentially unsuitable for the development of a stable transfection system that would require a high level expression of the exogenous gene (able to confer resistance to a selectable marker) [90,91].

Therefore, the following transfection constructs developed for *Babesia* spp. used the *rap-1* intergenic regions for its termination signals [6,94,95,97]. Studies of *rap-1* locus organization have been conducted for several *Babesia* spp., revealing that the *rap-1* family consists in multiple gene copies arranged in tandem and separated by IG regions [134]. In *B. ovis*, the *rap-1a* locus is partially described, pointing towards the existence of 5 closely linked genes (*Bo60.1 – Bo60.5*) with an apparent high sequence conservation between genes *Bo60.1-60.4* and lower similarity to *60.5* [135].

Currently, three *rap-1* genes in *Babesia* spp. designated *rap-1a, rap-1b* and *rap-1c* are described. However, it appears that this feature is confined to a phylogenetic group comprising *B. motassi-like* and *B. bigemina* [136,137]. In *B. ovis* all the copies have been assigned as *rap-1a* with two different gene types within this designation (*rap-1a*60-1-4 and the type *rap-1a*60-5). In a recent study it was verified that *RAP-1a* sequences from *B. s*p. Xinjiang formed a clade with *B. ovis, B. bovis* and *B. orientalis* clustering with all the babesial RAP-1a type sequences [136]. Recent work with *B. gibsoni* transfection indicated that *B. bovis* 3' *rap-1a* has cross-species function even though the interchangeability was not tested [100]. Exploring *B. ovis rap-1* locus can give a better insight in its structure, important not only to use the IG region for transcription termination but also because RAP-1 proteins have been proposed as a potential candidate for the development of recombinant vaccines against babesiosis due to their implication in erythrocyte invasion and high immunogenicity [134,136]. Assessing the number of *rap-1* copies in *B. ovis* genome would also provide more information to study this region regarding its importance as a virulence factor [138].

1.3.3. Selectable markers

The development of a stable transfection system requires the identification of an appropriate genetic marker in order to select the transgenic parasites, i.e., a selectable marker [2,6]. The firsts reports of stable transfection in apicomplexan parasites, namely *Plasmodium,* used the **WR99210/ *human dihydrofolate reductase* (*hdhfr)* selection system** [139]. The enzyme *dihydrofolate reductase* (*dhfr*) has been a target for several anti-malarial drugs since its inhibition depletes the parasite reduced folate pool, blocking *de novo* thymidylate biosynthesis by thymidylate synthase, and eventually preventing DNA synthesis [140]. The 4,6-diamino-1,2-dihydro-1,3,5-triazine WR99210 is not available as anti-malarial drug due to its high toxicity, however it was used to successfully select transgenic *P. berghei* parasites harbouring the h*DHFR* gene, since this gene conferred resistance to the antifolate drug [141].

The first attempts to stably transfect *B. bovis* used the same strategy, however naturally resistant parasites to WR99210 emerged [6]. In fact, *B. bovis* contains a copy of the *dhfr* enzyme gene, required for synthesis of precursors of purines and thymidylate. Moreover, it was found out that the presence of polymorphic versions of this gene, such as the single amino-acid change present in *B. bovis dhfr* enzyme, renders the parasite resistant to antifolate drugs, namely WR99210 [13,140]. Interestingly, subsequent studies were able to select transgenic-*B. bovis* parasites and more recently, this strategy was used to select transgenic-*B. ovata* and transgenic-*B. gibsoni* [7,94,98,108].

The **blasticidin/*blasticidin-deaminase* (*bsd*) selection system** has been described for effective selection of transgenic-*Plasmodium* parasites and it was the first system used to select stably transfected *B. bovis* parasites [93,142]. Blasticidin S is a nucleoside antibiotic able to inhibit protein synthesis in both prokaryotes and eukaryotes [143]. For parasites infecting an anucleate host erythrocyte the drug concentration range is not restrained by toxic effects in the host cell [142,144]. Also, there are no *bsd* homologous genes in *B. bovis* genome and experimental evidence demonstrated that *B. bovis* was highly susceptible to blasticidin, supporting it as a good selection system [13,93]. In *B. bigemina* blasticidin-S was also used as a suitable selective inhibitory drug [96].

The previous reports support the evaluation of *B. ovis* sensitivity to WR99210 and blasticidin-S to assess the suitability of these selection systems.

1.3.4. Genome integration targets

The integration of exogenous DNA into a specific genome region depends on homologous recombination mechanisms, a process that consists in the exchange of genetic information between two identical DNA sequences [145]. The existence of different patterns of processing transfected genes has been proposed for *Toxoplasma*, *Plasmodium* and *Babesia*, as it heavily depends of their available DNA repair mechanisms [8,146]. Gene targeting in *Toxoplasma* parasites is innately ineffective due to the ability of randomly inserting the foreign DNA in different sites from the specifically targeted due the existence of a non-homologous end joining mechanism, a problem solved by knocking out *KU80*, an important component of the pathway [147]. In contrast, for *Plasmodium*, the process of exogenous DNA integration depends exclusively on homologous recombination mechanisms, as reviewed by Limenitakis and Soldati-Favre (2011) [101]. Even so, for *Plasmodium*, gene targeting is still quite inefficient due to the unique richness of A + T of its genome that impairs efficient exogenous DNA integration [104].

Babesia and *Plasmodium* both present homologous recombination mechanisms and the random insertion of exogenous genes appears to be rare, however *Plasmodium* parasites can use both single and double cross- over insertion recombinant mechanisms whereas for *Babesia* it is demonstrated in several transfection studies that gene insertion occurs exclusively through a double crossover mechanism [7,8,93,94,96,98,101,108,148] (**Figure 6**). Thus, *Babesia* spp.- specific transfection appears to be an efficient genetic manipulation technique [8].

The first report of a stable transfection system for *B. bovis* targeted one of the *ef-1α* gene copies and, several posterior transfection studies confirmed the successful and specific integration in this site [7,93,94,96,98,108]. Furthermore, the disruption of one of the two *ef-1α* gene copies by a double-crossed homologous recombination mechanism in several *Babesia* spp. did not affect parasite viability and the growth of the transfected isolates is comparable to the wild-type parasite [93,94,96,98]. An identical *ef-1α locus* arrangement in *B. ovis* genome is expected supporting the feasibility of using this *locus* for exogenous gene integration.

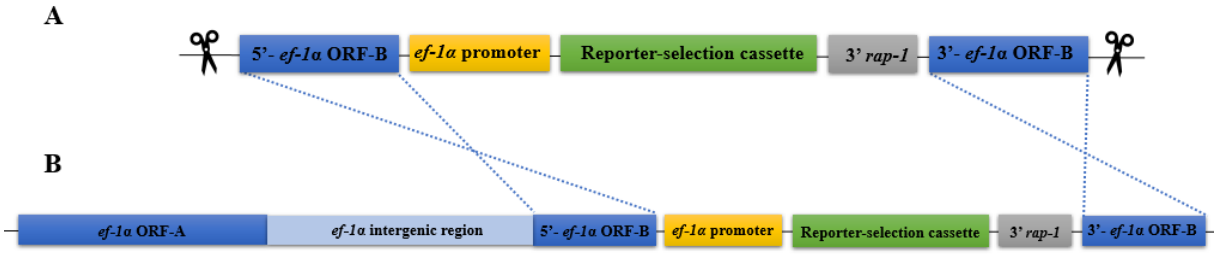

Figure 6. Schematic representation of a stable transfection construct integrating *elongation factor-1alpha* open reading frame-B through a double crossover recombination event. A. Stable transfection construct with *elongation factor 1α* (*ef-1α*) promoter driving expression of a cassette comprising a reporter gene and a selectable marker gene with 3' *rhoptry associated protein-1* (*3' rap-1*) as terminator **B.** Representation of the *ef-1α* locus in a transfected parasite line. ✂ represent enzyme restriction sites for plasmid linearization.

2. Objectives

The main goal of this project was the development of a stable transfection system driving expression of a fluorescent marker for *B. ovis*.

In order to achieve this goal, the following specific aims were established:

1. Identification of suitable regulatory elements in *B. ovis* genome and/or identification of their heterologous in other *Babesia* spp;

2. Construction of transient transfection plasmids with the previously selected promoter regions, driving expression of a reporter luciferase;

3. Evaluation of transfection process parameters, transfection efficiency and promoter activity;

4. Identification of a suitable selection system for *B. ovis* transfectants;

5. Construction and validation of a transient transfection system for *B. ovis* with the previously selected promoter driving expression of a fusion protein expressing the fluorescent and selectable maker genes;

This study represents a step forward in the development of methods for *B. ovis* genetic manipulation, an undoubtedly necessary tool to study this parasite basic biology, including its life cycle, the parasite interactions with host cells and virulence factors.

3. Materials and Methods

3.1. *In vitro Babesia ovis* cultures

B. ovis in vitro cultures were established in biosafety level 2 facilities at Instituto de Higiene e Medicina Tropical (IHMT), following an adapted protocol from Vega *et al* [84]. Briefly, cryopreserved *B. ovis* (Israeli strain) infected red blood cells (iRBC) at 8% parasitemia were used to initiate the culture. Parasites were cultured in 10% (vol/vol) defibrinated lamb erythrocytes (commercialized by Biorabbit, Lisbon, Portugal) maintained in a HEPES-buffered Medium 199 (1x) supplemented with Earle's Salts, L-glutamine and L-Amino acids (Gibco, Life technologies, Thermo Fischer Scientific, Waltham, Massachusetts, USA). Culture complete medium was further supplemented with 20% (vol/vol) lamb serum (Gibco, Thermo Fischer Scientific) and Antibiotic-Antimycotic (100x) at a final concentration of 0,05 µg/mL amphotericin, 20 U/mL penicillin and 20 µg/mL streptomycin (both from Gibco, Thermo Fischer Scientific), 1 mM L-cysteine-HCl, 2 mM bathocuproine-disulfonic acid and 25 mM *N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (all from Sigma-Aldrich, St. Louis, MO, USA) [73].

Vega y Martinez (VYMS) solution was used for washing and storage lamb erythrocytes for up to 5 weeks and the cryoprotectant used to freeze parasite culture was 20% polyvinylpyrrolidone-40 (PVP-40) (Sigma-Aldrich) in VYMS solution [149].

Parasite culture was maintained in a microaerophilic stationary phase atmosphere (5% CO_2, 2% O_2 and 93% N_2) at 37ºC using a modular incubation chamber, as described elsewhere [73]. In a daily basis, 80 % of the medium was replaced and culture parasitemia was monitored by preparation of thin blood smears stained with Hemacolor® Rapid staining of blood smear (EMD Millipore, Darmstadt, Germany). Intraerythrocytic parasites were observed under a 400x original magnification of a Motic BA210 LED trinocular compound microscope.

3.2. Evaluation of *Babesia ovis* sensitivity to WR9910 and blasticidin-S

B. ovis parasites were cultured in 1 mL of culture medium containing 10% lamb RBC (RBC suspension) in 24-well plates (Corning Costar, Corning, New York, USA). For WR9910 sensitivity assay, drug concentrations of 0.5, 1, 5, 10 and 50 nM were prepared from a 0,017 M stock solution of WR99210 (Sigma-Aldrich) in 100% dimethyl sulfoxide (DMSO), as done elsewhere [94]. Negative control was performed only with the drug vehicle to a final concentration of 0.1 % (v/v), the same DMSO concentration of the wells subjected to drug pressure[150]. For blasticidin-S, drug concentrations of 0, 1.2, 2.4, 4.8, 7.2, 9.6, 12, 14.4, 19.2, 24 and 28.8 µg/mL

were prepared from a 5 mg/mL blasticidin-S (Panreac AppliChem, ITW reagents, Barcelona, Spain) stock solution as done elsewhere[96].The drug vehicle, sterile water, was used as negative control.

The initial percentage of parasitized erythrocytes (PPE) for all the sensitivity assays was approximately 1% and it was achieved artificially by diluting the ongoing *B. ovis* culture with an appropriate volume of RBC suspension.

Parasites were cultured in triplicate for each drug concentration and 80% of the culture medium was replaced daily by fresh medium with the appropriate drug concentration. PPE was calculated at 24 h, 48h and 72h by examining at least 1000 RBCs of a prepared thin blood smear stained as described in **section 3.1**. To calculate the % of *B. ovis* growth inhibition and the 50 % inhibitory concentration (IC_{50}) the following formula was used, as done elsewhere [93]:

$$\frac{\text{Mean PPE in control wells-Mean PPE in blasticidin-S treated wells}}{\text{Mean PPE in control wells}} \; x\,100$$

3.3. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in the present study are listed in **Table 2**. *E. coli* was grown at 37ºC in Luria-Bertani (LB) broth with shaking or agar[151]. Super Optimal broth with Catabolite repression (S.O.C.) (2% tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was also used when appropriated. Supplementation with 100 µg mL^{-1} ampicillin (Panreac AppliChem) or 50 µg mL^{-1} kanamycin (Sigma-Aldrich) was carried out for transformants selection. Cell concentrations were estimated by measuring the optical density (O.D.) at 600nm (O.D.600) using a spectrophotometer (Biophotometer-Eppendorf).

Table 2. Strains and plasmids used in the present study.

Strains and plasmids	Description	Reference /Source
Escherichia coli		
JM109	*recA1 endA1 gur96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔM15]	Stratagene
TOP10F'	F′ [*lac*I^q, *Tn*10(Tet^R)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen

Strains and plasmids	Description	Reference /Source
Plasmids		
pLVX-TetOne™-Puro-Luc	Inducible Lentiviral expression vector of *Photinus pyralis* Luciferase reporter gene. Amp^R	Clontech
pCR™-Blunt I TOPO vector	A linearized and topoisomerase I-activated vector for 5 -minute benchtop ligations without ligase with a *ccdB* gene for positive selection of recombinants. Kan^R	Invitrogen
TOPO-RV1RV3	Cloning of the PCR product resultant from amplification with primers PR-Ef-ORF-B-RV1 and PF-Ef-ORF-B-RV3 in pCR™-Blunt I TOPO vector . Kan^R	This study
TOPO-rap	Cloning of *rhoptry associated protein* gene and 3' end in pCR™-Blunt I TOPO vector . Kan^R	This study
pBrfp-bsd	Plasmid with *B. bovis ef-1α* IG-B region driving expression of *rfp-bsd* gene, a fusion protein comprising a red fluorescent protein and blasticidin-S deaminase (selectable marker). Amp^R	[108]
pBef1αIG2	Plasmid with *B. ovata* IG-B region driving expression of Renilla Luciferase Reporter gene. Amp^R	[94]
pBbovis-luc	pBrfp-bsd with *B. bovis ef-1α* IG-B region driving expression of Luciferase reporter gene. Amp^R	This study
pBovata-luc	pBef1αIG2 with *B. ovata* IG-B region driving expression of Luciferase reporter gene. Amp^R	This study
pBS-luc	pBovata-luc without *B. ovata* IG-B region. Amp^R	This study
pBovata-rfp-bsd	Plasmid with *B. ovata* IG-B region driving expression of *rfp-bsd* gene. Amp^R	This study

3.4. Preparation and transformation of chemically and electrocompetent cells

Preparation of electrocompetent cells of *E. coli* was performed according to Smith *et al.*[152] Briefly, a culture reaching an OD_{600} of 0.6-0.7 in LB was centrifugated to form a cell pellet and washed 3 times with 10% ice-cold molecular biology grade glycerol (AppliChem). After the last centrifugation step, cells were concentrated 250-fold in 10% ice-cold glycerol. Electroporation was carried out in a Gene Pulser® Electroporation System (Bio-Rad) using a pulse of 1.8 KV, 25 µF and 200 Ω for 0.2 cm cuvettes or 1800 V, 25 µF and 200 Ω for 1 mm cuvettes.

Chemically competent cells were prepared following the Hanahan method [153]. Briefly, a culture with an OD_{600} of ≈ 0.4 in LB was pelleted, washed once with 50 mM ice-cold $CaCl_2$ solution and cells were concentrated 10-fold in ice-cold 50 mM $CaCl_2$-15% glycerol. All competent cells were frozen and stored at -80ºC until further use. For chemical transformation cells were centrifuged and resuspended in 50 mM CaCl2 before transformation which was carried out by heat shock, incubating cells with the appropriate DNA, on ice for 30 min and then placing them in a 42ºC water bath for exactly 2 min.

3.5. *Babesia ovis* DNA extraction

Parasite culture was centrifuged for 5 min. at 200 G, infected-RBCs (iRBCs) pellet was resuspended in 500 µg/mL trypsin in phosphate buffered saline buffer (PBS) and incubated at 37ºC for 20 min. Complete medium was added in a 1:1 proportion to the pellet and mechanical lysis of erythrocytes was performed by ten passages through a 26-gauge needle. The suspension was after centrifuged for 5 min/1500 G, to retrieve released *B. ovis* in the supernatant. Genomic DNA extraction was proceeded using NZY Blood gDNA Isolation Kit (NZYTech, Lisboa, Portugal), following manufacturers' instructions and genomic DNA eluted in 50 µL of manufacturers' provided elution buffer.

3.6. PCR, enzymatic restriction and purification

DNA fragments were amplified by PCR using the NZYTaq 2x Green Master Mix (NZYTech), unless high fidelity PCR products were required. For the latter, DNA polymerases with proofreading activity were used: *Pfu DNA Polymerase* (Promega, Madison, Wisconsin, USA), NZYSpeedy Proof DNA polymerase (NZYTech, Lisboa, Portugal) or iProof™ High-Fidelity DNA Polymerase (Bio-Rad, CA, USA). PCR amplifications were carried out accordingly to manufacturers' instructions in standard thermocyclers (Applied Biosystems SimpliAmp or Bio-Rad T100 Thermal Cycler). The amplification products were analysed by electrophoresis in 0.8 to 1.2% (w/v) agarose gels containing GreenSafe Premium (NZYTech) or ethidium bromide and using 0.5X TBE as electrophoresis buffer. Gels were analysed under UV light and pictures were acquired using ChemiDoc system (Bio-Rad). The fragments size was estimated by comparing with DNA ladders (NZYDNA Ladder VI, VII and VIII, NZYTech; or GeneRuler 1 kb DNA Ladder, Thermo Scientific) run along with DNA samples.

DNA fragments obtained from PCR amplification or enzymatic restriction products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany), NZYGelpure Kit (NZYTech) or GRS PCR & Gel Band purification Kit (Grisp, Porto, Portugal), following

manufacturers' instructions. Isolation and recovery of DNA fragments or digested vectors run in low-melting point agarose gel (Seakem™ LE agarose, Lonza, Basel, Switzerland) stained with ethidium bromide were purified using NZYGelpure Kit (NZYTech).

Plasmid DNA extraction and purification was performed with NZYMiniprep kit (NZYTech), following the protocol recommended by the manufacturer. DNA was eluted in the minimal volume of sterile distilled water and stored at -20ºC. Restriction enzymes (FastDigest®, Fermentas) and T4 DNA ligase (New England Biolabs) were used according to supplier's instructions.

DNA concentration and purity (analysis of $A_{260/A280}$ ratio) was estimated using a ND-1000 Spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, Waltham, MA).

3.7. Amplification, cloning and sequencing

To construct plasmids TOPO-RV1RV3 and TOPO-rap, amplicons generated by PCR with primers PR-Ef-ORF-B-RV1/ PF-Ef-ORF-B-RV3 and Rap1α-Dissimilar-FW/ Rap1-3-RV, respectively, conjointly with *Pfu DNA polymerase* (Promega) were cloned into the pCR™-Blunt I-TOPO vector (Invitrogen, Thermo-Fisher Scientific). The recombinant plasmids were transformed into TOP10 chemically competent *E. coli*, following the Zero Blunt TOPO PCR Cloning kit instructions. Ten colonies were selected for colony screening PCR and the inserted fragment was checked with Supreme NZYTaq II *DNA polymerase* (NZYTech, Lisboa, Portugal) using the appropriate primers. A single colony was then picked and grown overnight in S.O.C medium containing kanamycin (50 µg/mL), at 37ªC with shaking (180 rpm). Plasmid DNA extraction and purification was carried out as described in **section 3.6** and presence of the insert was further analyzed by restriction analysis using *Bam*HI and *Xho*I restriction enzymes. The recombinant vectors containing the fragments of interest were sent to Sanger sequencing at GATC Biotech (Ebersberg, Germany) with the universal primers **M13 Forward (-20)** and **M13 Reverse**.

3.8. Screening for suitable regulatory regions in *Babesia ovis* genome

The 5' flanking region of the *actin* gene was explored using the available sequences of *B. bovis* and *B. bigemina actin* gene (GenBank accession numbers AK441690 and XM_012912903). Basic Local Alignment Search Tool (BLAST) was used to search sequences in EuPathDB: The Eukaryotic Pathogen Genomics Resources (https://eupathdb.org/eupathdb/) to retrieve the upstream non-coding region of *B. bovis* and *B. bigemina actin* gene. Searches with the MEME Suite 5.0.2 used motif length constraints of 5 bp to 15 bp, searching both

strands at the DNA level for a maximum of 10 motifs with an E-value cut-off of 0.05 assuming zero or one occurrence of a motif as has been done previously [120,154] The search was complemented with the use of FIMO version 5.0.2 program to search for particular motif occurrences [155].

The sequences from *B. bovis*, *B. bigemina* and *B. divergens* (GenBank accession numbers: DQ322644, KT439182 and LK934712, respectively) were used to explore the *B. ovis ef-1-α* locus. The *rap-1* locus was also analyzed for transcription termination using the *B. ovis rap-1* locus partially described (GenBank accession numbers: M91169, M91170, M91172, M91173, M91174 and M91176)[135]. Primers aiming the sequencing of unknown regions and the understand of *B. ovis rap-1* locus organization were manually designed.

The oligonucleotides used for all the strategies aiming amplification and sequencing of *B. ovis 5' actin, ef-1-α* and *rap-1* locus were evaluated using the OligoAnalyzer from Integrated DNA technologies (https://eu.idtdna.com/calc/analyzer) and are listed in **table 3.**

3.8.1. Methodologies for *Babesia ovis elongation factor-1α* and *rhoptry associated protein-1* intergenic regions locus assembly

The strategies developed to assemble *B. ovis ef-1α* locus were based on an initial analysis of *ef-1α-B* gene sequences from *B. bovis*, *B. bigemina* and *B. divergens* using the bioinformatics tool: Clustal Omega multiple sequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/) [156]. A set of reverse primers (**PR-Ef-ORF-B-RV1**/ **PF-Ef-ORF-B-RV3)** were manually designed to take advantage of the head to head orientation for intergenic region PCR amplification, as done elsewhere [157]. Also, to proceed to a PCR-mediated genome walking method the set of primers **PF-ef-ORFB/PR-Ef-ORF-B** were manually designed to amplify part of *ef-1α-B* gene **(Figure 7)** [156].

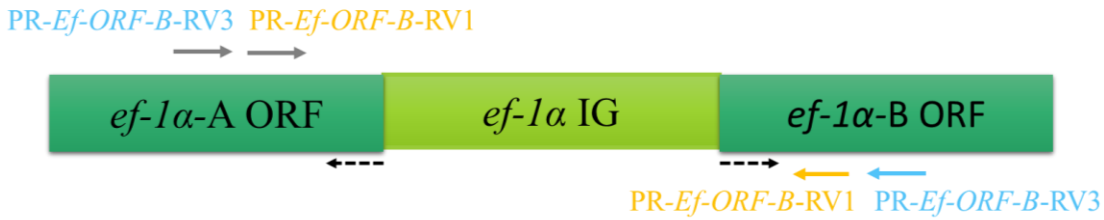

Figure 7. Expected organization of *Babesia ovis elongation factor-1alpha* locus based on *Babesia bovis* sequence. Primers PR-Ef-ORF-B-RV1 and PR-Ef-ORF-B-RV3 hybridization sites in *ef-1α-B* gene are represented with yellow and blue arrows, respectively. Grey arrows represent primers hypothetical hybridization sites in *ef-1α*-A gene. Expected orientation of the open reading frames (ORF) is shown with black dashed arrows. (not for scale). **Adapted from:** Suarez *et a*l., 2006 [91].

PR-Ef-ovis-specific was designed to hybridize in *B. ovis ef-1α-B gene* sequenced region for PCR amplification with non-specific primers targeting the *ef-1α-B* 5' flanking region (**PF-**

Ef-IG1 and **PR-Ef-IG3**). This strategy intended to amplify the promoter region upstream *ef-1α-B* gene (**Figure 8**), as described elsewhere [158,159].

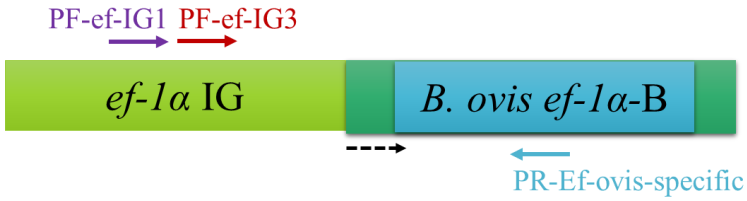

Figure 8. Scheme of the PCR-mediated genome walking method for *Babesia ovis elongation factor-1alpha* locus. A sequence specific primer, PR-Ef-ovis-specific, was designed from *ef-1α-B* gene known sequence (blue box) to generate a PCR product with non-specific primers designed from *B. bovis ef-1α* IG sequence (PR-ef-IG1 and PR-ef-IG3) that anneal in *B. ovis* genome under a low annealing temperature. Hybridization sites of primers are shown (not for scale). Expected orientation of the open reading frame (ORF) is shown with a black dashed arrow.

The approach to understand *B. ovis rap-1* locus, consisted in the design **Rap1α-Dissimilar-FW**, hybridizing in a region found dissimilar between the available *rap-1* gene copies sequences using the Clustal Omega multiple sequence alignment program [156] and a primer reverse hybridizing in *rap-1 60.5* 3' end, **Rap1-3-RV**.

Table 3. Primers used for amplification and sequencing of *Babesia ovis elongation factor-1alpha and rhoptry associated protein-1* locus

Primer name	Sequence (5'→3')
Primers used to amplify *Babesia ovis actin* gene	
PF-actin-gene	CCAAAGAACCCTGCCCTTAT
PR-actin-gene	GACGATGTTGGGTCCAGCCT
Primers used to amplify *Babesia ovis elongation factor-1alpha locus*	
PR-Ef-ORF-B-RV1	GCCAATAACGACCAAGTTAATGTG
PR-Ef-ORF-B-RV3	ACAACAAGCATAGCAACATCGG
PF-Ef-ORF-B	CAAGTACGCCTGGGTTTTGGAC
PR-Ef-ORF-B	CGGTACGCTTGTCCATACGG
PF-Ef-IG1	TATCAAAAACACACAATACT
PR-Ef-ovis-specific	CACGTTCACGCTCACTCTTCAGC
PF-Ef-IG3	CACGTAATAAATGAGAT
Primers used to amplify *Babesia ovis rhoptry associated protein 1* locus	
Rap1α-Dissimilar-FW	CGCACCAAGGACTTCTTC
Rap1-3-RV	GAATTCGTTAAGCTCGGTGA
Primers for sequencing	
M13 Forward (-20)	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC

3.9. Transient transfection plasmid constructs

The ability of *B. bovis* and *B. ovata ef-1α* IG-B regions to function as heterologous promoters was evaluated through expression of a *firely luciferase* (*luc*) gene in *B. ovis*. For this, two transient transfection constructs were developed, the pBbovis-luc and pBovata-luc.

The pBrfp-bsd (**Table 2**) (kindly provided by professor Masahito Asada) was used as backbone for development of pBbovis-luc construct. Linearized vector containing only *B. bovis ef-1α* IG-B sequence was obtained by enzymatic restriction with *Eco*RV and *BamH*I and was isolated and purified from a 0.8% low-melting point agarose gel as described in **section 3.6**. The *luc* gene was amplified by PCR from pLVX-TetOne™-Puro-Luc (**Table 2**) with primers **PF-luc-*Eco*RV/ PR-luc-*Bgl*II** and the 3' *rap-1* terminator sequence with primers **PF-bovis-rap-1-*Bgl*II/ PR-bovis-rap-1-*Bam*HI** (**Table 4**). Both purified PCR products were digested with *Bgl*II restriction enzyme, followed by a ligase reaction. After enzyme inactivation (65 ºC for 10 minutes), PCR amplification of *luc*-3'*rap-1* cassette with primers **PF-luc-*Eco*RV/ PR-bovis-rap-1-*Bam*HI** was performed as done elsewhere [160]. The amplified fragment was then digested with *Eco*RV and *Bam*HI restriction enzymes and ligated with the linearized vector, producing pBbovis-luc (**Figure 9**).

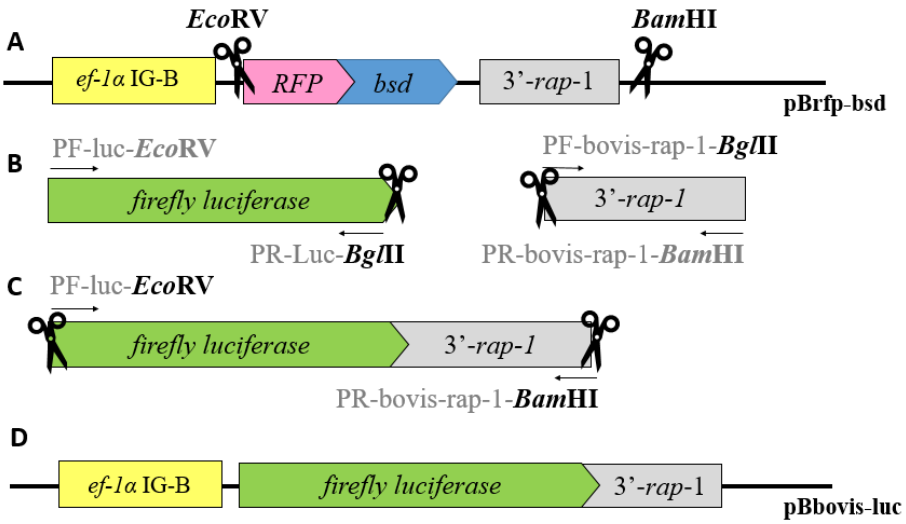

Figure 9. Schematic diagram representing the development of the transient transfection plasmid Bbovis-luc. (A) Schematic representation of pBrfp-bsd, the backbone for construction of pBbovis-luc. ✂ represent the restriction sites of the enzymes used for construct development. *ef-1α* IG-B - flanking region of the *ef-1α* gene from *B. bovis*; *RFP-bsd*, gene coding a fusion protein comprising a Red fluorescent protein and Blasticidin-S Deaminase*; 3'-rap-1*, 3' flanking region of the *rhoptry associated protein-1* gene. **(B)** *Firefly luciferase* gene and *3' rap-1* were amplified by PCR with the represented primers and digested with *Bgl*II restriction enzyme. **(C)** PCR amplification of the *firefly luciferase-3'rap-1* cassette was performed with the primers shown and the cassette was digested. **(D)** Ligation of the digested vector with *firefly luciferase-3'rap*-1 cassette to originate pBbovis-luc.

The pBef1αIG2, a plasmid carrying the *B. ovata ef-1α* IG-B region (**Table 2**) (kindly provided by professor Masahito Asada) was used as backbone for development of pBovata-luc. First, it was digested with *Eco*RV and *Eco*RI restriction enzymes and the resulting linearized vector was isolated and purified from a 0.8% low-melting point agarose gel as described in **section 3.6.** The *luc* gene was PCR amplified with **PF-luc-*Eco*RV/ PR-luc-*Eco*RI** and later digested with the same restriction enzymes to allow ligation of *luc* gene to the digested vector between *B. ovata Ef-1-α* IG-B sequence and 3′ *rap-1a* terminator region resulting in pBovata-luc (**Figure 10**).

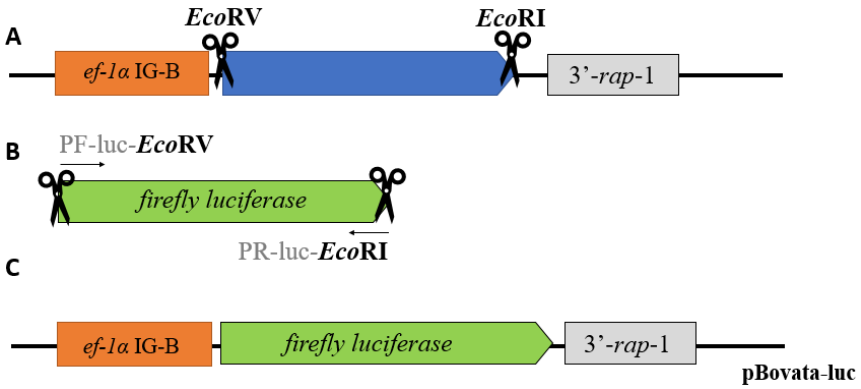

Figure 10. Schematic diagram representing the development of the transient transfection plasmid Bovata-luc. (A) Schematic representation of the plasmid used as backbone for construction of pBovata-luc. ✂ represent the restriction sites of the enzymes used for construct development. *ef-1α* IG-B- flanking region of the *ef-1α*-B gene from *B. ovata*; *3'-rap-1*, 3' flanking region of the *rhoptry associated protein-1* gene. **(B)** *Firefly luciferase* gene was amplified by PCR with the represented primers and digested with shown restriction enzymes. (**C)** Ligation of the digested vector with *firefly luciferase* originating pBovata-luc.

A promoterless plasmid pBS luc was also developed to be used as negative control in assays to measure the luciferase activity. Briefly, pBovata-luc was digested with *Hind*III restriction enzyme to excise the promoter sequence. After vector isolation and purification from a 0,8% low-melting agarose gel as described in **section 3.6**, it was re-circularized by ligation (**Figure 11**).

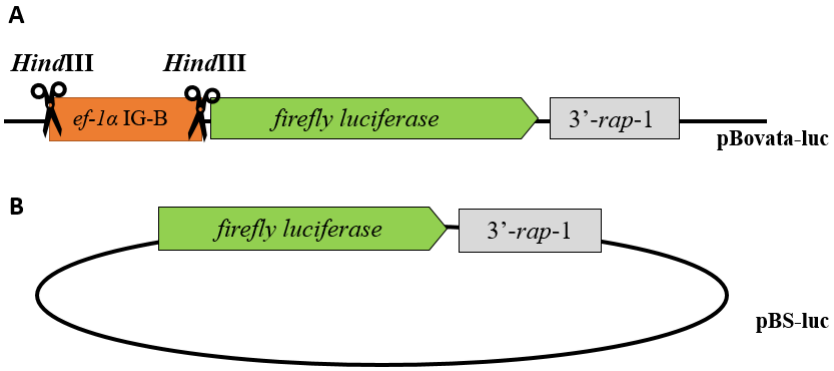

Figure 11. Schematic diagram representing the development of the promoterless plasmid, pBS-luc. (A) pBovata-luc was digested with represented restriction enzymes. ✂ represents enzymes restriction sites. (**B**) pBS-luc was generated by re-circularization.

3.10. Plasmid construct validation and preparation for transfection

Plasmid constructs were transformed into *E. coli* JM109 competent cells and selection of the recombinant strains was performed by colony screening PCR using appropriate primer sets. Plasmids from colonies showing the expect PCR product were extracted and purified as described in **section 3.6.** Afterwards, enzymatic restriction was performed for construct confirmation and all the correct insertion was confirmed by Sanger sequencing at StabVida (Lisbon, Portugal) with the appropriate primers (**Table 4**).

To obtain a higher amount of recombinant plasmids, a midiPrep was prepared using the Qiagen Plasmid Midi Kit (Qiagen) following manufacturers' instructions. Plasmid DNA concentration was assessed as mentioned in **section 3.6.** to guarantee transfection of the desired plasmid DNA amount throughout transfection experiments.

Table 4. Primers used for development of transfection constructs and to assess transfection efficiency.

Primer Name	Sequence (5'→3')	PCR product size (bp)
Primers for transient transfection constructs		
PR-luc-***Bgl*II**	GCG**AGATCT**TTACAATTTGGACTTTCCG	1653
PF-luc-***Eco*RV**	GCC**GATATC**ATGGAAGACGCCAAAAAC	1653
PR-luc-***Eco*RI**	CGC**GAATTC**TTACAATTTGGACTTTCCGC	1653
PF-bovis-rap-1-***Bgl*II**	GCC**AGATCT**GATGAGATGCGTTTATAATGG	1294
PR-bovis-rap-1-***Bam*HI**	CGC**GGATCC**CCTACGAACGATATGTCAAAG	1294
PR-luc-seq. [b]	CGCCGCCGTTGTTGTTTTGG	
PF-luc-seq. [b]	CAGCCCATATCGTTTCATAGC	
M13F-pUC (-40) [b]	GTTTTCCCAGTCACGAC	
Primers for development of a stable transfection construct		
PF-prom-ovata-***Sal*I**	CTA**GTCGAC**CACTCATTTAGATTGCGAC	724
PR-prom-ovata-***Hind*III**	CTG**AAGCTT**CTTGTTTAAGGTTTAACGATAG	724
PF-5ef-ORF-***Xho*I**	GTT**CTCGAG**TTCAAGTACGGCCTGGGT	483
PR-5ef-ORF-***Sal*I**	CTA**GTCGAC**TTGCCCTTGTACCATGGC	483
PF-3ef-ORF-***Not*I**	A**GCGGCCGC**GACCTTGGTCGAGGCCCTCGAC	495
PR-3ef-ORF-***Sac*I**	GTC**GAGCTC**TTGTCCATACGGCAGGTGATCTCGTCG	495
Primers for qPCR to evaluate transfection efficiency		
PF-BoSPD *	TAATGACGCAGACCTGATGG	141
PR-BoSPD *	GTTTGATCACCCTCGGAAAC	141
PF-qPCR-luc	GCTGGGCGTTAATCAGAGAG	151
PR-qPCR-luc	GTGTTCGTCTTCGTCCCAGT	151

Restriction enzyme sites are shown in **bold.** ***Erster *et al*., 2016 [161]

3.11. Procedures for transfection

B. ovis iRBCs with PPE approximately 6% were centrifuged at 1200 rpm for 10 min. The supernatant was discarded, and cell pellets were washed twice, first with PBS and then with cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 25 mM HEPES, pH 7.6, 2 mM EGTA, 5 mM $MgCl_2$, 100 µg/ml bovine serum albumin, and 1 mM hypoxanthine), a protocol adapted from Asada *et al* [94].

For transfection, two different protocols of iRBC preparation were conducted accordingly to the transfection kit in use. For the Basic Parasite Nucleofector™ Kit 2 (Lonza), 20 µg of circular plasmid in elution buffer were mixed with Nucleofector™ Basic Solution for Parasites to a final volume of 100 µL, which was used to resuspend 100 µL of washed parasite-iRBCs. For the Human T-cell Nucleofector™ Kit (Lonza), 20 µg or 50 µg of circular plasmid in elution buffer were mixed with cytomix buffer to make up a volume of 50 µL and added to 50 µL of human T-cell buffer. Similarly, this mixture was used to resuspend 100 µL of washed parasite-iRBCs. The plasmid pmaxGFP™, provided in the Human T-cell Nucleofector™ Kit (Lonza), was used as positive control of transfection. Evaluation of successful transfection was performed by imaging *B. ovis* transiently expressing GFP under 400x original magnification of a Nikon eclipse 80i fluorescence microscope with Nikon DS-Ri1 camera (Nikon Europe, Amsterdam, Netherlands) and appropriate filter (GFP).

Parasite-iRBCs were transfected with circular plasmids in a Nucleofector 2b Device with a v-024 program (Amaxa Biosystems, Cologne, Germany) and were immediately transferred to 1 ml culture containing 10 % ovine RBCs. Due to constrains related to buffer availability only one transfection was performed for each condition. Transfected parasites were cultured in 24-well plates as described previously in **section 3.1**.

3.12. Chemiluminescence quantification to evaluate promoter activity and optimization of transfection process

Assays to measure luciferase activity were performed to evaluate differences in transfection outcomes between two transfection kits, Basic Parasite Nucleofector™ Kit and Human T-cell Nucleofector™ Kit, and between transfection with either 20 µg or 50 µg of plasmid DNA.

After optimization of the transfection conditions by the previous evaluation, luciferase reporter assays were performed to quantify luciferase expression promoted by *ef-1α-B* intergenic regions from *B. bovis* and *B. ovata* (**Figure 12**).

Transient transfections were conducted by introducing 20 µg of each promoter plasmid expressing *luc* gene, pBbovis-luc and pBovata-luc and a promoterless plasmid for negative control, pBS-luc (detailed description in **section 3.11**). For evaluation of promoter activity at least three independent transfections of each plasmid construct were performed. Luciferase reporter assays were performed in triplicate at 24h, 48h and 72h after transfection using ONE-Glo™ Luciferase Assay System (Promega). Following manufactures' instructions, to 100 µL of transfected *B. ovis* culture, the same amount of ONE-Glo™ Luciferase Assay Reagent was added. To ensure complete cell lysis, this mixture was kept for 5 minutes at room temperature before reading luminescence. Luciferase assays were performed in triplicates in a Corning® Costar 96-well white plate (Corning Costar) and luminescence was measured for a 2 sec. integration interval in a GloMax-Multi+ Detection System (Promega).

3.13. Transfection efficiency by quantitative real-time PCR

Transfection efficiency was evaluated through qPCR. DNA extraction was performed from 80 µL of transfected *B. ovis* culture (with pBbovis-luc, pBovata-luc and pBS-luc) 24h post-transfection, following the protocol described in **section 3.5 (Figure 12).**

A qPCR was developed to assess the quantity of *luc* and *B. ovis surface protein D (Bo*SPD*)* genes in the transfected *B. ovis* parasites using the sets of primers presented in **table 3**, following the minimum information for publication of qPCR experiments (MIQE) guidelines [162]. The qPCRs were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, CA, USA) and the cycling conditions comprised an enzyme activation step at 95 ºC for 5 min followed by 35 cycles of denaturation at 95ºC for 10 s and annealing/extension at 51ºC for 15 s and 60 ºC for 15 **s** for *luc* and 54,5 ºC for 30 s for *BoSPD*. To confirm amplification specificity for *luc* and *Bo*SPD, a melting curve was run (55 °C–95 °C; 0.5 °C/s melt rates). Reactions were performed in triplicate to a final volume of 10 µL**,** with 5 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), for *luc* each primer at 500 nM and for *BoSPD* each primer at 800 nM, 1 µL of DNA and nuclease-free water to achieve the final volume. Negative controls were prepared with no template.

A gBlocks® Gene Fragment (Integrated DNA Technologies, Leuven, Belgium) was synthetized for both *luc* and *Bo*SPD each with 151 and 141 bps, respectively. Standard curves were prepared for each gene with 10-fold serial dilutions of the synthetized gBlocks® Gene Fragment and obtained by plotting the threshold cycle (C_q) versus the natural log of concentration (ng/µl). These curves were used to determine qPCR reactions efficiency (analytical sensitivity) and for quantification of *luc* and *Bo*SPD. Copy numbers of *luc* and

BoSPD genes were calculated based on the following formula, as done previously [163], assuming that *Bo*SPD gene only has one copy *per* genome, where *X* represents the amount of amplicon (ng):

$$\text{Copy number} = \frac{X \text{ ng x } 6{,}0221 \text{ x } 10^{23} \text{ molecules/mols}}{\text{Amplicon molecular weight x } 1 \text{ x } 10^{9} \text{ ng/g}}$$

Transfection efficiency is presented as the ratio of plasmid/genome copies (*luc/ Bo*SPD copies), as done elsewhere [90,95].

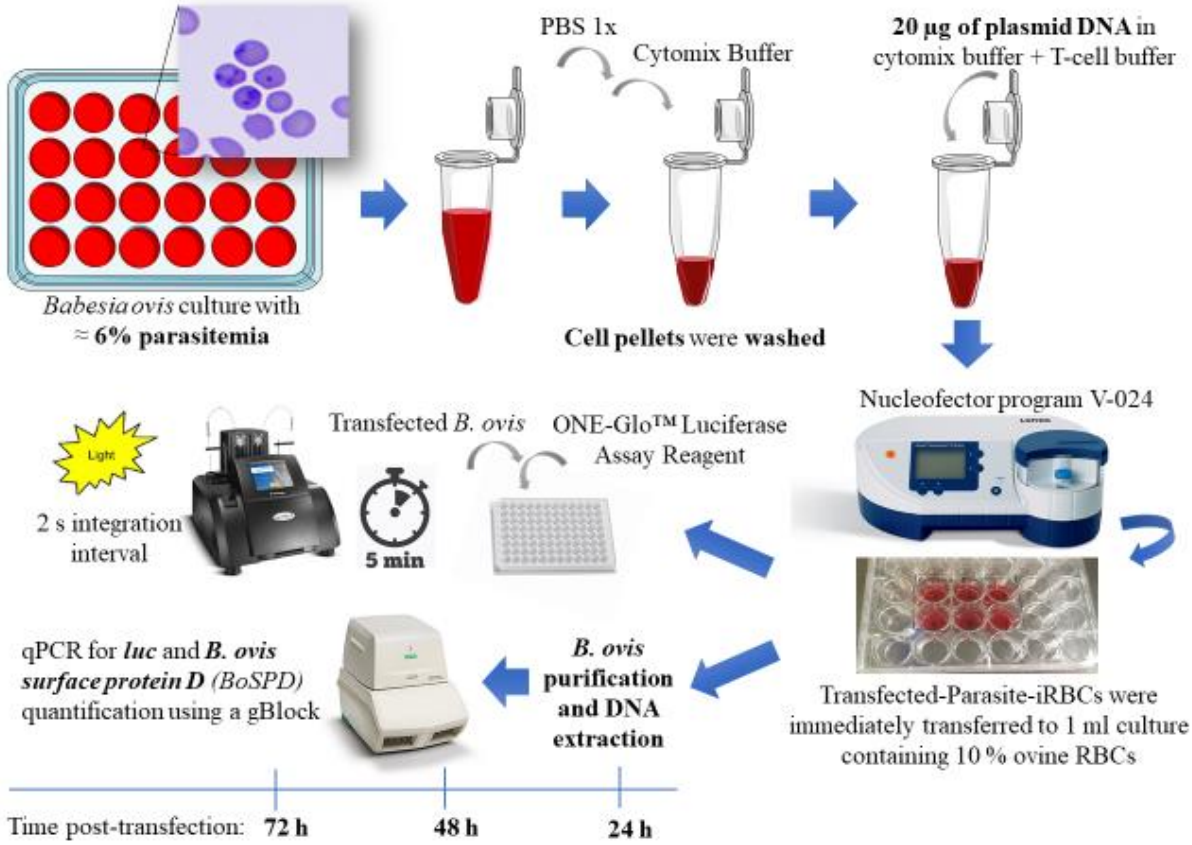

Figure 12. Schematic representation of the methodologies used to evaluate activities of *elongation factor-1alpha* intergenic region-B candidate promoters from *Babesia bovis* and *Babesia ovata* in *Babesia ovis*.

3.14. Development of a stable transfection plasmid construct

Brfp-bsd plasmid was digested with *Sal*I and *Hind*III restriction enzymes to excise the pre-existing *B. bovis ef-1α* promoter region as described in **section 3.6.** *B. ovata ef-1α*-B promoter region was amplified from pBef1αIG2 with primer set **PF-prom-ovata-*SalI*/ PR-prom-ovata-*HindIII* (Table 4)** and ligated to the previously digested vector creating pBovata-rfp-bsd. Confirmation of *B. ovata ef-1α-B* promoter region integration into the plasmid vector

was performed by a colony screening PCR, followed by Sanger sequencing with **M13F-pUC (-40) primer** at StabVida (Lisbon, Portugal).

The construct was used for transient transfection using optimized procedures and RFP expression was validated by fluorescence microscopy. Briefly, images of *B. ovis* transiently expressing RFP were obtained under 100x original magnification of a Nikon eclipse 80i fluorescence microscope with Nikon DS-Ri1 camera (Nikon Europe, Amsterdam, Netherlands) and appropriate filter (Texas Red).

PCR from previously extracted *B. ovis* gDNA (DNA extraction process in **section 3.5**) was used for generation of *ef-1α* flanking regions for stable transfection. The primer sets used were **PF-5ef-ORF-*Xho*I** and **PR-5ef-ORF-*Sal*I** for *B. ovis ef* 3' region (expected 495 bp amplicon); **PF-3ef-ORF-*Not*I** and **PR-3ef-ORF-*Sac*I** for the 5' region of *B. ovis ef* (expected 483 bp amplicon). Sanger sequencing of the PCR products was performed at StabVida (Lisbon, Portugal).

3.15. Statistical Analysis

The luciferase activities and ratio of plasmid/ "genome copies" values were plotted using Microsoft Excel as the mean ± standard deviation, calculated from the triplicate samples. The one-way ANOVA analysis was used for data comparison ($P<0.05$) using GraphPad Prism6.

4. Results and Discussion

4.1. Screening and identification of suitable regulatory regions in *B. ovis* genome

Brief note regarding *B. ovis* genome: one of the goals of the present study was the identification of regulatory regions in *B. ovis* genome, thus in line with the reasons described previously in the introduction section (1.3.2 and 1.3.4) the 5' flanking region of the *actin* gene, the *ef-1α* and *rap-1* locus, were chosen. However, recently we became aware that *B. ovis* Israeli strain genome had been sequenced but information is not public. Promptly the group responsible for this work, in the person of Doctor William Weir from University of Glasgow, was contacted for a collaborative effort. Therefore, in the following section all the strategies performed to amplify, sequence the aforementioned regions and the results will be discussed. When appropriate, results will be compared with information provided by Doctor William Weir.

4.1.1. Isolation of the 5' flanking region of the *actin* gene

The first strategy was focused in PCR amplification of part of *B. ovis actin* gene using primers designed to amplify a 1246 bp fragment within *B. bovis actin* gene. A range of primer annealing temperatures between 59ºC and 55ºC was tested and it was observed that for all the temperatures a PCR product with approximately 1200 bp in size was originated, indicating the amplification of a product coincident in size to the expected in *B. bovis* (**Figure 13**). After sequencing the amplified product, we obtained a 952 nucleotides sequence.

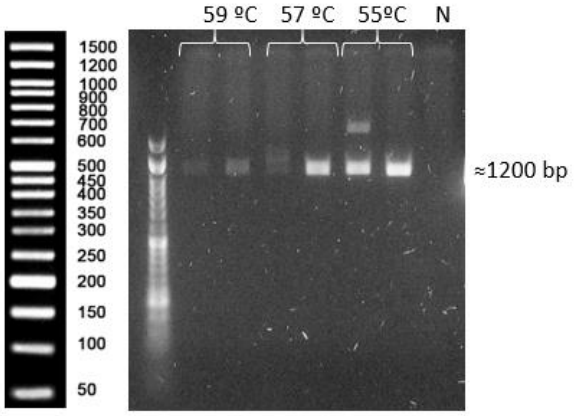

Figure 13. Amplification of part of *Babesia ovis actin* gene. Electrophoresis of the PCR products resultant of amplification of part of *B. ovis actin* gene in a 1.2% (vol/vol) agarose gel. From left to right: NZYDNA Ladder VI, gradient of annealing temperatures from 59ºC to 55ºC, PCR negative control (N).

The alignment of the previously obtained sequence against *B. ovis* Israeli strain draft genome allowed to retrieve the complete *B. ovis actin* gene sequence (1131 bp) (**Appendix I.1**

and I.2). A BLAST search of the obtained sequence against available sequences in the NCBI Database from Babesiidae family was performed revealing the highest homology with *B. bovis actin* gene sequence (89% identity) (**Table 5**). A brief analysis showed a single continuous 1131 bp ORF and comparison of the amino acid sequence against available sequences in the UniProt Database revealed 100% homology with *B. sp. Xinjiang* and *B. bovis* Actin protein showing that its highly conserved between *Babesia* spp. **(Table 6)**.

Table 5. Babesiidae annotated sequences producing significant alignments with *Babesia ovis actin* gene sequence.

Description	Maximum Score	Total Score	Percent Query Coverage	E Value	Maximum Percent Identity	GenBank Accession Number
Babesia bovis BBOV_I000300 mRNA for *actin*, complete cds, clone: XBBk019120, strain: Texas	1160	1160	99%	0	89%	AK441690.1
Babesia bigemina actin, putative partial mRNA	1018	1018	99%	0	86%	XM_012912903.1
Babesia gibsoni actin mRNA, complete cds	941	941	99%	0	85%	XM_012912903.1

Table 6. Babesiidae annotated protein sequences producing significant alignments with *Babesia ovis actin* translated sequence.

Protein name	Score	E Value	Identity	Accession Number
Actin (*Babesia sp. Xinjiang*)	1958	0.0	100.0%	A0A1X1BPP4
Actin (*Babesia bovis*)	1958	0.0	100.0%	A7AX51
Actin, putative (*Babesia bigemina*)	1940	0.0	98.7%	A0A061D645
Actin (*Babesia gibsoni*)	1915	0.0	96.5%	Q5EF72

Attempts to amplify and sequence the 5' flanking region failed but it was possible to access this region after retrieving 1000 bp upstream *B. ovis actin* coding sequence **(Appendix I.3)** from *B. ovis* genome sequencing project. A discontiguous megablast was performed with this sequence and, for a query coverage of 40% (507 to 908 bp), it was found a 68% homology with the sequence correspondent to the promoter region of *B. bovis* strain *actin* gene **(Appendix I.4)**. Analysis of the 5' *act* was performed using the sequence obtained in the present study and three *actin* promoter sequences from *B. bovis*, *B. ovata* and *B. gibsoni* (GenBank accession numbers: MF598086, LC101765 and MF598081). Using MEME bioinformatic tool, it was possible to detect 3 motif sequences almost co-localized between sequences from *B. ovis* and *B. bovis* (GCCCCAGCG, CACATGTGCT and GGCACCGCTG $p<0.0001$). The TSS initiator-

like motif, TYAYWWW, was searched using FIMO program and, for *B. bovis* this motif was found located 152 bp before the *actin* coding sequence, which is the median length described between the TSSs to the coding sequence 5' end (p<0.0001) for this species [113]. This motif is also present in *T. parva* genome, usually, 3 bp upstream of TSSs, but seems that other motifs might have similar functions [120]. This consensus sequence was found in *B. ovis* but located in a region that apparently should not have a role as TSS [123,164].

Transfection using *actin* promoter was achieved for *B. ovata*, *B. bovis* and *B. gibsoni* with 2300 bp, 1800 bp and 1455 bp, respectively, of the 5' flanking region of the gene [7,94,100] suggesting that essential transcriptional factors, bind in the most proximal 5´ region. Though, *cis*-regulatory elements play a significant role in promoter activity, imposing a positive or negative effect as it was described for *T. gondii* [119,165]. Some recently reported results have shown that *B. gibsoni 5'act* was able to drive more expression of the reporter gene than the homologous promoter region in *B. bovis* [127]. Since no homology exists between the two sequences, the presence of motifs in *B. gibsoni 5'act* lacking in *B. bovis 5'act*, for which *B. bovis* transcriptional factors had more affinity, can be suggested [112]. Therefore, having in mind, that almost half of the *B. ovis* 5'*act* region has a 68% homology with *B. bovis*, it would be interesting to conduct a transient transfection in both parasites to check promoter activity of this most "conserved" portion in comparison with the all *B. ovis* 5'*act* sequence available.

Transfection studies in apicomplexan parasites have been used as a proof of concept for the functionality of several promoter regions and even to identify interspecies activity of some them, however the application of such genetic manipulation methods for the identification of key motifs that define the promoter sequences are urgently required [2].

4.1.2. Exploring *elongation factor 1-alpha* locus

The *ef-1α* locus organization in *B. bovis* and *B. bigemina* comprises two identical *ef-1α* genes, designated *ef-1α-A* and *ef-1α-B*, arranged in a head to head orientation, separated by an intergenic region [91,95]. Two strategies were developed, both based in the design of primers hybridizing in *B. bovis ef-1α*-B gene in sites showing the lowest nucleotide divergence between three *Babesia* species.

4.1.2.1 A structure similarity-based strategy

The first strategy used to amplify *B. ovis ef-1α* IG region assumed an identical structure between *B. ovis* and *B. bovis ef-1α* locus. Therefore, it was expected that the designed

oligonucleotides would amplify fragments containing the intergenic region, with approximately 1500 bp, 1800 bp and 2100 bp in size (**Figure 14**).

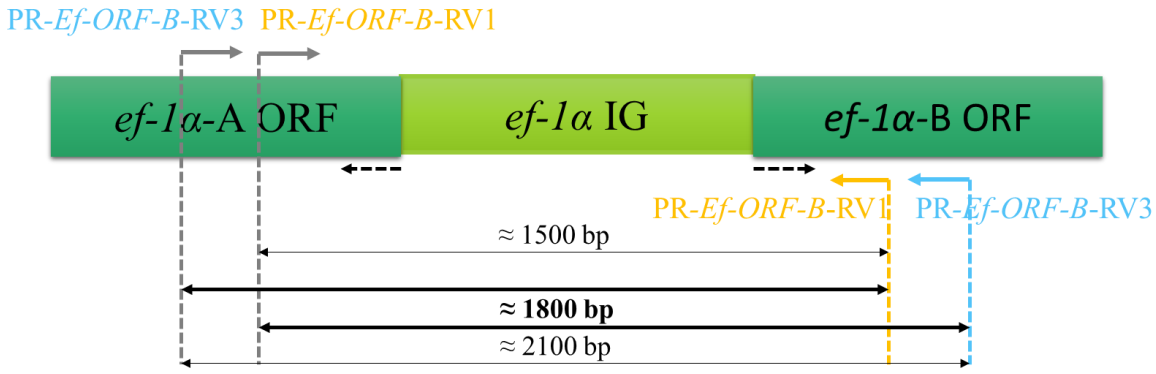

Figure 14. Expected amplicon sizes for PCR with primers PR-Ef-ORF-B-RV1 and PR-Ef-ORF-B-RV3. The amplicon size estimated assumes an identical structure between *B. ovis* and *B. bovis ef-1α* locus. Grey arrows represent primers hypothetical hybridization sites in *ef-1α-A* gene. Expected orientation of the open reading frames (ORF) is shown with black arrows (not for scale).

PCR experiments were carried out using a DNA polymerase with proofreading activity, and, of several *B. bovis* PCR products, amplicons with approximately 1000 bp, 1800 bp and 2000 bp were originated, which coincided in size to the expected (**Figure 14 and Figure 15A, lane *Bb***), validating the present approach. In *B. ovis*, one of the obtained amplicons, with approximately 1500 bp (**Figure 15A, lane *Bo***), was subjected to a re-amplification (**Figure 15B**) and selected for isolation (**Figure 15C**) due to its relative size proximity to the 1800 bp fragment amplified in *B. bovis.*

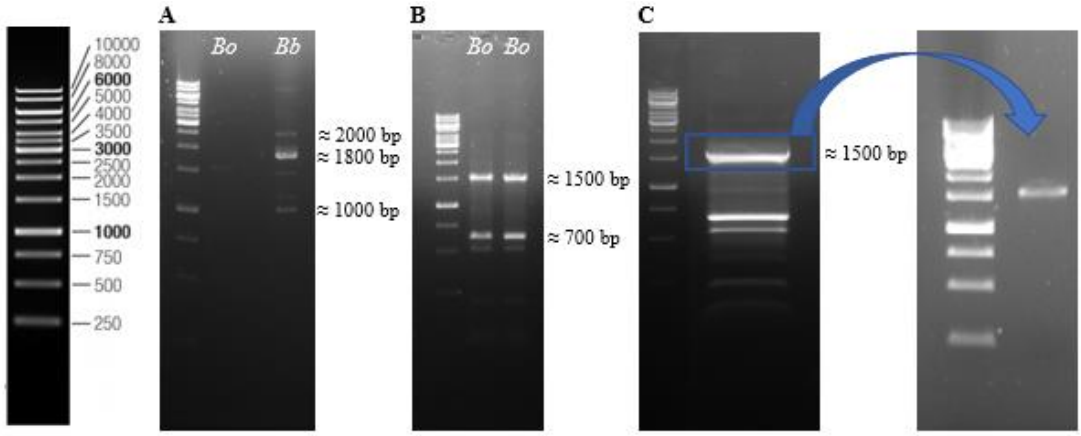

Figure 15. Amplification and isolation of a *Babesia ovis* 1500 base pairs (bp) size fragment. Electrophoresis of **(A)** the PCR products resultant of amplification with primers PR-Ef-ORF-B-RV1 and PR-Ef-ORF-B-RV3 having *B. ovis* (Bo) and *B. bovis* (Bb) gDNA as template, **(B)** re-amplification of the previously obtained PCR product and **(C)** isolation and purification of the 1500 bp fragment in a 0.8% (vol/vol) agarose gel. Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder shown in the left.

The previously obtained 1500 bp amplicon was cloned into the pCR™-Blunt I-TOPO originating TOPO-RV1RV3 and, after validating the presence of the fragment, it was sent to Sanger sequencing. A BLAST optimized to search for dissimilar sequences (discontiguous

megablast) was conducted for the sequences obtained (two products with 692 bp and 794 bp) **(Appendix II.1)**, revealing, for the highest coverage of the submitted sequences, a 68% nucleotide sequence identity with a *B. bovis* coding sequence for protein kinase domain containing protein **(Table 7)**.

Table 7. Babesiidae annotated sequences producing significant alignments with the cloned *Babesia ovis* 1500 base pairs in size sequence.

Description	Maximum Score	Total Score	Percent Query Coverage	Expect Value	Maximum Percent Identity	GenBank Accession Number
Babesia bovis BBOV_III003600 mRNA for protein kinase domain containing protein, complete cds, clone: XBBk019034, strain: Texas	230	460	84%	1^{-55}	68%	AK441676.1
Babesia bovis protein kinase domain containing protein (BBOV_III003600) mRNA, complete cds	228	457	84%	5^{-55}	68%	XM_001611442.1
Babesia bigemina protein kinase domain containing protein, putative partial mRNA	72.5	145	14%	8^{-8}	75%	XM_012913984.1

PCR experiments were conducted using a *Taq II DNA polymerase* and for the same annealing temperature tested before (54.4 ºC), PCR products with a size closer to 2000 bp were observed. Lowering the annealing temperature until 49.5 ºC favored the amplification of these products whose size were closer to the 1813 bp and 2118 bp as obtained in *B. bovis* **(Figure 16)**. Sequencing of amplicons failed. Also, attempts were performed to replicate this result using a DNA polymerase with a proofreading activity without success.

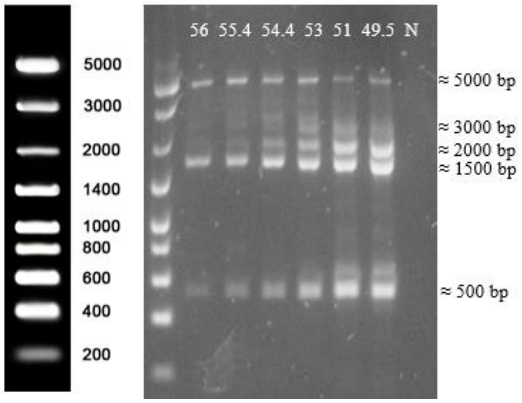

Figure 16. Attempts for *Babesia ovis elongation factor 1-alpha* Intergenic region amplification. Electrophoresis of the PCR products resultant of amplification with primers PR-Ef-ORF-B-RV1 and PR-Ef-ORF-B-RV3 having *B. ovis* gDNA as template in a 1.2% (vol/vol) agarose gel. From left to right: NZYDNA Ladder VIII, gradient of annealing temperatures from 56ºC to 49.5 ºC, PCR negative control (N).

The failure to amplify *B. ovis ef-1α* IG region could be related with the lack of specificity of the designed primers for the intended hybridization sites in *B. ovis*. However, the number of mismatches or even a single mismatch at the primer 3' end, that affect PCR more dramatically than at other positions, [166,167] should only result in a reduction of amplification efficiency, still allowing amplification to occur. Using the of *B. ovis ef-1α* sequence **(Appendix III.2)** retrieved from the *B. ovis* genome this hypothesis was tested with PR-Ef-ORF-B-RV1 presenting a single nucleotide mismatch (3 bps from the 3' end) and PR-Ef-ORF-B-RV3 having two nucleotides diverging from the template sequence, one located at 7 bps from the 5´end and other at 2 bp from the 3´end.

Overall, these results may point to a different locus organization, contrary to other *Babesia* spp. This locus structure is identical between *B. bovis*, *B. bigemina* and *B. ovata* with synteny among the two *ef-1α* genes reported to be conserved among *B. bigemina*, *B. bovis* and *Plasmodium* parasites [91,95]. Therefore, more studies should be conducted to understand *B. ovis ef-1α* locus structure.

4.1.2.2 A PCR-mediated genome walking method

Since the previous strategy failed to amplify the fragment of interest, a second approach, consisting in an adapted PCR-mediated genome walking method was performed. Here, PCR was carried out with a sequence-specific primer, plus non-specific primers hybridizing in the unknown region [158,168,169170]. Hence, there was the need to first amplify and sequence the region adjacent to the unknown region, the *ef-1α* gene.

Based on the previous used alignment, two primers were designed to amplify part of *B. ovis ef-1α* gene, correspondent to an amplicon of approximately 947 bp in size. One amplicon was obtained with the expected size **(Figure 17)**.

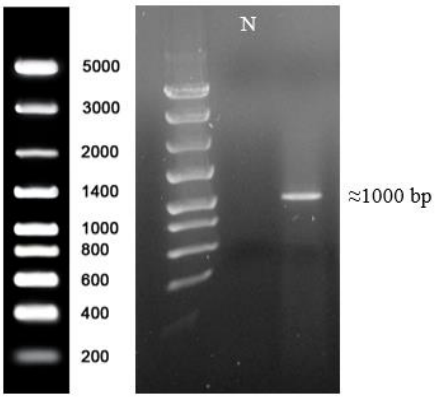

Figure 17. Amplification of part of *Babesia ovis elongation factor-1alpha* gene. Electrophoresis of the PCR product resultant of amplification of part of *B. ovis ef-1α* gene in a 1.2% (vol/vol) agarose gel. From left to right: NZYDNA Ladder VIII, PCR negative control (N).

The 940 bp product was sequenced and a BLAST search revealed a high nucleotide sequence homology with *B. ovata* and *B. bigemina* (92%). Alignment of the partial *ef-1α* gene obtained with the *B. ovis* draft genome was also performed and complete *B. ovis ef-1α* gene sequence is now described **(Appendix III.1 and III.2) (Table 8).**

Table 8. Babesiidae sequences producing significant alignments with *Babesia ovis elongation factor-1alpha* gene sequence.

Description	Maximum Score	Percent Query Coverage	Expect Value	Maximum Percent Identity	Accession Number
Babesia ovata strain Miyake transcript	1948	100%	0.0	92%	BOVATA_017780-t37_1*
Babesia bigemina strain Puerto Rico *elongation factor 1 alpha A* and *elongation factor 1 alpha B* genes, complete cds	1946	100%	0.0	92%	XM_012911267.1
Babesia divergens genome assembly 454hybrid_PBjelly, scaffold Contig2	1853	100%	0.0	90%	LK934712.1
Babesia bovis elongation factor 1-alpha (BBOV_IV010630) mRNA, complete cds	1817	100%	0.0	90%	XM_001610934.1
Babesia microti strain R1 elongation factor EF-1 alpha subunit	820	100%	0.0	74%	BMR1_03g03490-t32_1*

*Accession number correspondent to transcript identification (Gene ID) in EuPathDB genomic resources database

After designing a reverse primer hybridizing in *B. ovis ef-1α* gene sequenced region **(PR-Ef-ovis-specific)** and two non-specific primers targeting the least divergent sites between *B. bovis* and *B. bigemina ef-1α-B* 5' flanking region (**PF-Ef-IG1** and **PR-Ef-IG3**), PCR experiments were conducted. PCR with **PF-Ef-IG1/ PR-Ef-ovis-specific** and **PR-Ef-IG3/ PR-Ef-ovis-specific** were expected to result in amplicons with approximately 1200 bp and 900 bp, respectively **(Figure 18).**

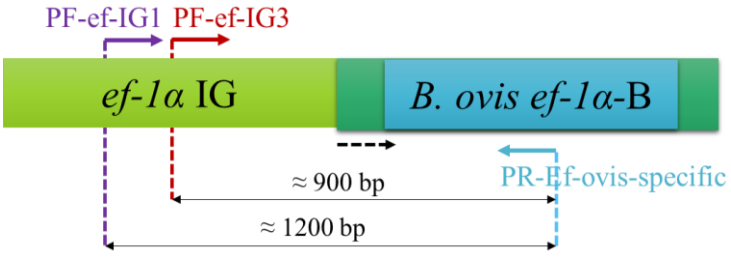

Figure 18. Expected amplicon sizes for PCR with primers PR-Ef-IG1/ PR-Ef-ovis-specific and PR-Ef-IG3/ PR-Ef-ovis-specific. The amplicon size estimated assumes an identical structure between *B. ovis* and *B. bovis ef-1α* locus. Hybridization sites of primers are shown (not for scale). Expected orientation of the open reading frame (ORF) is shown with a black arrow.

Among several *B. ovis* PCR products, amplicons with approximately 1400 bp were obtained with **PF-Ef-IG1/ PR-Ef-ovis-specific (Figure 19A).** Also, using the second primer

set, amplicons of similar size were amplified, and lowest annealing temperatures (43.9 and 41.4ºC) resulted in the amplification of DNA fragments with 900 bp **(Figure 19B)**.

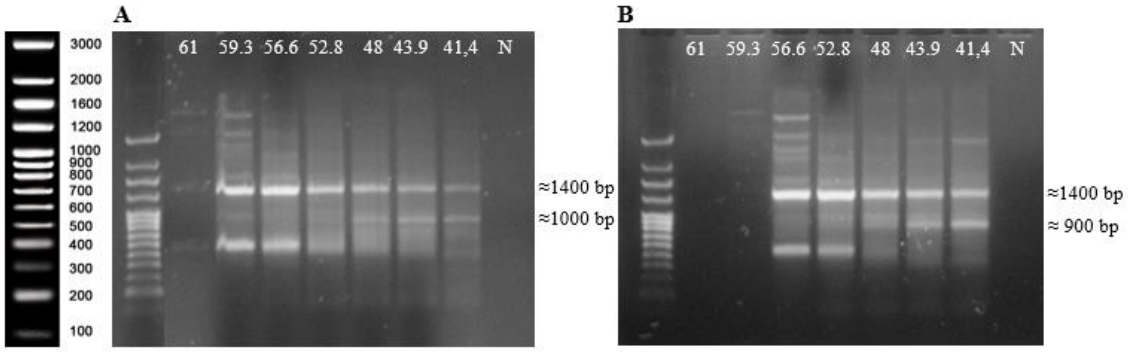

Figure 19. Attempts for amplification of *Babesia ovis elongation factor-1α* 5' flanking region. Electrophoresis of the PCR products resultant of **(A)** amplification with PF-Ef-IG1/ PR-Ef-ovis-specific and **(B)** amplification with PF-Ef-IG3/ PR-Ef-ovis-specific in a 1.2% (vol/vol) agarose gel. From left to right: NZYDNA Ladder VII, gradient of annealing temperatures from 61ºC to 41.4 ºC, PCR negative control (N).

The PCR products obtained with 1400 bp and 900 bp in size were isolated, purified and sent to sequencing with the **PR-Ef-ovis-specific** however, only a 332 bp sequence was retrieved **(Appendix IV.1),** which did not align with the *B. ovis ef-1α* gene sequence. Considering that the **PR-Ef-ovis-specific** was designed to hybridize 185 bp downstream *ef-1α* gene start codon, the absence of this "anchor" sequence indicated that no *B. ovis ef-1α* 5' flanking region was amplified. Moreover, a discontiguous megablast was performed for the obtained sequence and no homology was found against Babesiidae sequences **(Appendix IV.2)**.

PCR experiments with **PF-Ef-IG3/ PR-Ef-ovis-specific** were conducted at lower annealing temperatures (40ºC to 36 ºC) increasing the amount of the 900 bp PCR product **(Figure 20)**. After isolation and purification, fragment was sent for sequencing in both 5´and 3´direction. Alignment of the sequences demonstrated that a single continuous 745-nucleotide fragment had been sequenced (**Appendix V.1**).

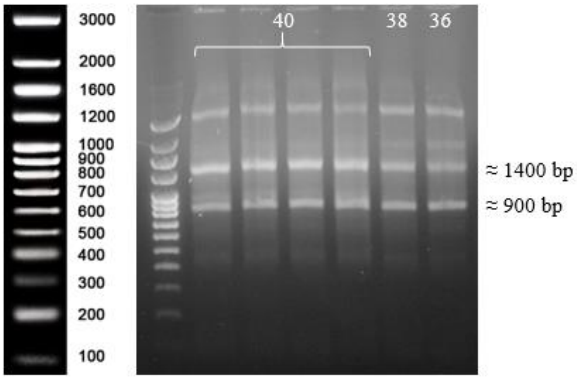

Figure 20. Attempts for amplification of *Babesia ovis elongation factor-1alpha* 5' flanking region with annealing temperatures between 36ºC and 40ºC. Electrophoresis of the PCR products resultant of amplification with PF-Ef-IG3/ PR-Ef-ovis-specific in a 1.2% (vol/vol) agarose gel. From left to right: NZYDNA Ladder VII, gradient of annealing temperatures from 40 ºC to 36 ºC, negative control (N).

Analysis showed that again no *B. ovis ef-1α* gene sequence was present in the amplicon. A discontiguous megablast was also conducted and demonstrated homology with *B. bovis* and *B. bigemina* hypothetical protein partial mRNA sequence. Herein, only alignments that have a coverage of the query sequence above 30% are shown **(Table 9)**.

Table 9. Babesiidae sequences producing significant alignments with the *Babesia ovis* obtained sequences from the genome walking PCR method.

Description	Maximum Score	Total Score	Percent Query Coverage	Expect Value	Maximum Percent Identity	GenBank Accession Number
Babesia bovis BBOV_IV001750 mRNA for hypothetical protein, complete cds, clone: XBBk027088, strain: Texas	88.7	88.7	42%	4^{-13}	68%	AK442121.1
Babesia bigemina genome assembly Bbig001, chromosome : V	82.4	82.4	56%	2^{-11}	66%	LK391711.1
Babesia bigemina hypothetical protein, conserved partial mRNA	77.0	77.0	55%	7^{-10}	65%	XM_012914811.1
Babesia divergens genome assembly 454hybrid_PBjelly, scaffold Contig3	68.0	68.0	44%	3^{-7}	66%	LK934713.1

Additionally, the amplified 745 bp sequence blasted against sequences available in the EuPathDB genomic resources database. Homology with sequences from *B. ovata* strain Miyake, *B. bigemina* strain BOND and *B. bovis* T2Bo were found (different sequence IDs seen in **table 10**) which did not co-localize with the *ef-1α* locus in these species [171].

Table 10. Babesiidae sequences producing significant alignments with the *Babesia ovis* obtained sequences from the genome walking PCR method and their genomic location.

Organism	Sequence ID which contains the region of alignment	Score	E-value	Sequence ID which contains the *ef-1α* locus
***Babesia ovata* strain Miyake**	BDSA01000005	78.8	3^{-12}	BDSA01000002
***Babesia bigemina* strain BOND**	LK391711	64.4	6^{-8}	LK391707
***Babesia bovis* T2Bo**	AAXT01000004	48.2	0.004	AAXT01000002

More studies need to be performed to clarify these results. Contrasting with the nucleotide sequence homology of *ef-1α* coding sequence between *Babesia* spp. **(Table 8)** and

other Apicomplexa, the *ef-1α* IG region is not conserved between species and between *B. bovis* and *B. bigemina* the IG sequence was considered unrelated [91,95].

The alignment of *B. ovis ef-1α* gene produced only one hit against *B. ovis* genome sequence which could indicate the presence of only one copy. However, only 155 bp upstream the gene were retrieved, indicating a possible sequencing gap in this region **(Appendix V.II)**.

4.1.2.1.1 Analysis of *Babesia ovis Elongation factor 1-alpha*

A brief analysis to *B. ovis ef-1α* ORF shows that encodes a protein of 448 amino acids with a predicted molecular weight of 49.31 Da, equal in size with homologues from *B. bovis*, *B. bigemina* and *B. sp. Xinjiang*, having the highest amino acid similarity with the latter (96.7%) (**Table 11**). Moreover, amino acid sequence analysis reveals the conservation of all putative guanosine 50-triphosphate (GTP) binding regions **(Figure 21)**, essential for the GTP-dependent binding of aminoacyl-tRNA to ribosomes during protein biosynthesis, also reported to conserved in *T. parva* and *P. falciparum* [91,95,172,173]. This suggests that this protein probably has an identical function between these apicomplexan parasites, being a key component of the protein translation mechanism. Moreover, it also indicates that to consider *B. ovis ef-1α* gene as a target for genome integration of a stable transfection system the presence of the two copies is essential to ensure parasite survival.

Table 11. Babesiidae Elongation factor 1-alpha protein sequences producing significant alignments with *Babesia ovis.*

Organism	Score	E value	Identity	UniProt Accession number
Babesia sp. Xinjiang	2268	0.0	96.7%	A0A1X1BH95
Babesia ovata	2250	0.0	95.3%	A0A2H6KBC5
Babesia bigemina	2250	0.0	95.3%	A0A061D9W5
Babesia bovis	2245	0.0	95.5%	Q2LEY6
Babesia microti (strain RI)	2026	0.0	85.9%	A0A0K3ARN2

```
B.ovis          MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKESTDMGKGSFKYAWVL 60
B.sp.Xinjiang   MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKESTDMGKGSFKYAWVL 60
B.ovata         MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKESTDMGKGSFKYAWVL 60
B.bigemina      MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKESTDMGKGSFKYAWVL 60
B.bovis         MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKESTDMGKGSFKYAWVL 60
B.microti       MPKEKTHINLVVIGHVDSGKSTTTGHLIYKLGGIDKRTIEKFEKDSSEMGKSSFKYAWVL 60
                ********************************************:*::***.******** 
                                GTP BS1
B.ovis          DKLKSERERGITIDITLWKFETSKYYYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAEA 120
B.sp.Xinjiang   DKLKSERERGITIDITLWKFETAKYYYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAEA 120
B.ovata         DKLKSERERGITIDITLWKFETGKYYYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAEA 120
B.bigemina      DKLKSERERGITIDITLWKFETGKYYYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAEA 120
B.bovis         DKLKSERERGITIDITLWKFETTKYYYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAEA 120
B.microti       DKLKSERERGITIDITLWKFETQKYEYTVIDAPGHRDFIKNMITGTSQADVAMLVVPAES 120
                ********************** ** ********************************: 
                                GTP BS2
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```
B.ovis          GGFEAAFSKEGQTREHALLAFTLGVRQIICAINKMDKCDYKEDRYSEIQKEVQGYLKKVG 180
B.sp.Xinjiang   GGFEAAFSKEGQTREHALLAFTLGVKQIICAINKMDKCDYKEDRYSEIQKEVQGYLKKVG 180
B.ovata         GGFEAAFSKEGQTREHALLAFTLGVKQIICAINKMDKCDYKEDRYSEIQKEVQGYLKKVG 180
B.bigemina      GGFEAAFSKEGQTREHALLAFTLGVKQIICAINKMDKCDYKEDRYSEIQKEVQGYLKKVG 180
B.bovis         GGFEAAFSKEGQTREHALLAFTLGVKQIICAINKMDKCDYKEDRYSEIQKEVQGYLKKVG 180
B.microti       GGFEAAFSKEGQTREHALLAFTLGVKQMIVAINKMDSCQYKEDRYMEIFKEVQQYLKKVG 180
                ************************:*:* ******.*:****** ** **** ******

                                          GTP BS3
B.ovis          YNTDKVPFVAISGFMGDNMVERSTNMPWYKGKTLVEALDMMEPPKRPVDKPLRLPIQGVY 240
B.sp.Xinjiang   YNIEKVPFVAISGFMGDNMVERSTNMPWYKGKTLVEALDMMEPPKRPIDKPLRLPLQGVY 240
B.ovata         YNIEKVPFVAISGFMGDNMVERSTNMPWYKGKTLVEALDMMEPPKRPVDKPLRLPLQGVY 240
B.bigemina      YNIEKVPFVAISGFMGDNMVERSTNMPWYKGKTLVEALDMMEPPKRPVDKPLRLPLQGVY 240
B.bovis         YNIEKVPFVAISGFMGDNMVERSTNMPWYKGKTLVEALDQMEPPKRPVDKPLRLPLQGVY 240
B.microti       YKVESVPFVAISGFHGDNMVEKSTNMPWYKGKTLVEALDQMEPPKRPVEKPLRLPLQSVY 240
                *: :.********* ******:***************** *******::******:*.**

B.ovis          KIGGIGTVPVGRVETGQLKAGMVLTFAPNPITTECKSVEMHHEVVEVAYPGDNVGFNVKN 300
B.sp.Xinjiang   KIGGIGTVPVGRVETGQLKAGMVLTFAPNPITTECKSVEMHHEVVEVASPGDNVGFNVKN 300
B.ovata         KIGGIGTVPVGRVETGQLKAGMVLTFAPNPITTECKSVEMHHEVIDVASPGDNVGFNVKN 300
B.bigemina      KIGGIGTVPVGRVETGQLKAGMVLTFAPNPITTECKSVEMHHEVIDVASPGDNVGFNVKN 300
B.bovis         KIGGIGTVPVGRVETGMLKAGMILTFAPNPITTECKSVEMHHETVEVAYPGDNVGFNVKN 300
B.microti       KIGGIGTVPVGRVETGQLKAGMIITFAPTGLTTECKSVEMHHEVVEVASPGDNVGFNVKN 300
                **************** *****::****. :************.::** ***********

B.ovis          VSTSDIRSGHVASDSKNDPAKAAVSFSAQVIVLNHPGNIKAGYTPVVDCHTAHVSCKFDE 360
B.sp.Xinjiang   VSTSDIRSGHVASDSKNDPAKAAVSFSAQVIVLNHPGTIKAGYCPVVDCHTAHVSCKFDE 360
B.ovata         VSTSDIRTGHVASDSKNDPAKAAVSFTAQVIILNHPGTIKAGYSPVVDCHTAHISCKFDE 360
B.bigemina      VSTSDIRTGHVASDSKNDPAKAAVSFTAQVIILNHPGTIKAGYSPVVDCHTAHISCKFDE 360
B.bovis         VSTSDIRSGHVASDSKNDPAKAAVSFTAQVIVLNHPGTIKAGYCPVVDCHTAHISCKFEE 360
B.microti       VSVKDIKRGNVASDSKNDPAKEATSFSAQVIVLNHPGTIKAGYSPVVDCHTAHIACKFES 360
                **..**: *:*********** *.**:****:*****.***** *********::***:.

B.ovis          ITCRMDKRTGKSLEENPKSIKNGDAAIVTLKPCKPMVVESFTEYAPLGRFAVRDMKQTVA 420
B.sp.Xinjiang   LTSRMDKRTGKALEENPKTIKNGDAAMVTLKPCKPMVVESFTEYAPLGRFAVRDMKQTVA 420
B.ovata         ITSRMDKRTGKALEENPKTIKNGDAAMVVLKPCKPMVVEAFTEYAPLGRFAVRDMKQTVA 420
B.bigemina      ITSRMDKRTGKALEENPKTIKNGDAAMVVLKPCKPMVVEAFTEYAPLGRFAVRDMKQTVA 420
B.bovis         ITSRMDKRTGKSLEENPKTIKNGDAAMVVLKPMKPMVVESFTEYAPLGRFAVRDMKQTVA 420
B.microti       LDTRIDKRTGKTLEENPKTIKNGDAAMVTMKPNKPMVVETFTDYAPLGRFAVRDMRQTVA 420
                :  *:******:******:*******:*.:** ******:**:************:****

B.ovis          VGVIKSVEKKEPGSSAKITKSAQKAAKK 448
B.sp.Xinjiang   VGVIKSVEKKEPGSSAKVTKSAQKAAKK 448
B.ovata         VGVIKSVEKKEPGSSAKITKSAQKASKK 448
B.bigemina      VGVIKSVEKKEPGSSAKITKSAQKASKK 448
B.bovis         VGVIKSVEKKEPGSSAKVTKSAQKAAKK 448
B.microti       VGIIKAVEKKDPSSA-KVTKSAVKAGKK 447
                **:**:****:*.*: *:**** **.**
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Figure 21. Alignment of Elongation factor 1-alpha protein sequences from *Babesia* species. Comparison between *Ef-1α* protein sequences from *B. ovis*, *B. sp. Xinjiang* (A0A1X1BH95), *B. ovata* (A0A2H6KBC5), *B. bigemina* (A0A061D9W5), *B. bovis* (Q2LEY6) and *B. microti* (A0A0K3ARN2). Three conserved EF-1a regions with known guanosine 50-triphosphate (GTP)-binding site function (GTP BS 1, 2 and 3) are shown in bold.

4.1.3 Exploring the *rhoptry associated protein 1* locus

The approach to understand *B. ovis rap-1* locus consisted in the design of a forward primer hybridizing in a region found dissimilar between the available *rap-1* gene copies sequences and a reverse primer hybridizing in the 3'end of the last known *rap-1* gene copy.

PCR led to the amplification of a PCR product with approximately 2000 bp in size **(Figure 22).** The 2000 bp amplicon was cloned into the pCRTM-Blunt I-TOPO and, after validating the presence of the fragment, it was sent to Sanger sequencing. From the 2000-nucleotide amplicon only 953 bp were sequenced. All the sequenced product aligned completely with the sequence already available (GenBank accession number: M91169) **(Appendix VI.1)**.

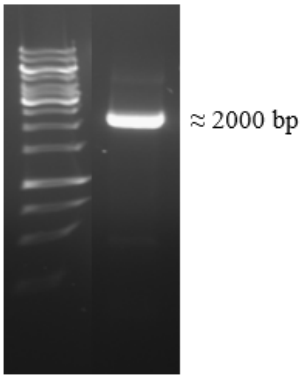


Figure 22. Amplification of *Babesia ovis rhoptry associated protein 1* gene and 3' end. Agarose gel electrophoresis at 0.8 % (vol/vol) of the PCR product resultant of amplification with Rap1α-Dissimilar-FW and Rap1-3-RV. DNA marker: NZYDNA Ladder VII.

Recently it was possible to align this obtained sequence against *B. ovis* Israeli strain, retrieving a sequence that might correspond to the *rap 1* locus sequence, however further studies are required to elucidate de locus organization. The intergenic region has already been sequenced which will be useful for the development of constructs with *B. ovis* autologous regulatory regions.

4.2. Transient plasmids construction

Being aware of the recognized inter-species activity of the *ef-1α* promoters among *Babesia* spp., pBbovis-luc and pBovata-luc were constructed with *B. bovis* and *B. ovata ef-1α* IG-B driving expression of a reporter protein luciferase, respectively. In addition, a promoterless plasmid was also developed to use as negative control in assays to measure the luciferase activity.

4.2.1. Development of plasmid with *Babesia bovis elongation factor-1alpha* intergenic region-B driving expression of a reporter luciferase

The pBrfp-bsd (**Table 2**) was used as backbone for development of plasmid pBbovis-luc. First, the plasmid was restricted with *EcoR*V and *BamH*I removing the RFP-BSD and 3'*rap* regions. The remaining plasmid, a fragment of 3671 bp containing the *B. bovis ef-1α* IG-B sequence was extracted from the gel. In a second step a *luc*-3'*rap-1* cassette was constructed to

replace the previously excised terminator region and insert the desired reporter luciferase sequence. For this, the *luc* gene was amplified from pLVX-TetOne™-Puro-Luc (**Table 2**) and the 3'*rap* region from pBrfp-bsd by PCR, originating products with approximately 1600 bp and 1300 bp, very near to the expected 1653 bp and 1295 bp, respectively (**Figure 23**).

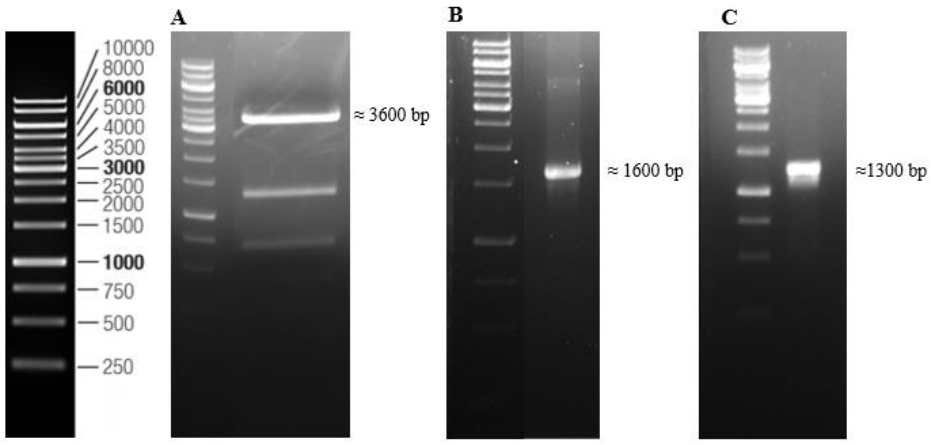


Figure 23. Digestion of plasmid Brfp-bsd and amplification of *firefly luciferase* (*luc*) gene and *rhoptry associated protein*-1 terminator region (*3'rap-1*). Electrophoresis of the **(A)** products of plasmid Brfp-bsd restriction digestion with *EcoR*V and *Bam*HI restriction enzymes, **(B)** PCR product resultant of *luc* gene amplification with pLVX-TetOne™-Puro-Luc as template and **(C)** PCR product resultant of *3' rap-1* amplification having pBrfp-bsd as template in a 0.8 % (vol/vol) agarose gel. Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific).

The presence of the *Bgl*II restriction site at the 3' end of *luc* and 5' end of *rap* 3' allowed the ligation of the two fragments after digestion of the purified PCR products with *Bgl*II. To ensure the success of the ligation, the reaction was performed using 1:1, 2:2, 3:2 and 4:3 for *luc* : *rap* 3' molar ratios. After heat inactivation of the T4 DNA ligase, PCR amplification of the *luc*-3'*rap-1* cassette was performed. An agarose gel electrophoresis was performed showing no noteworthy differences in the quantity of PCR product resultant from amplification with different ligation reactions **(Figure 24).**

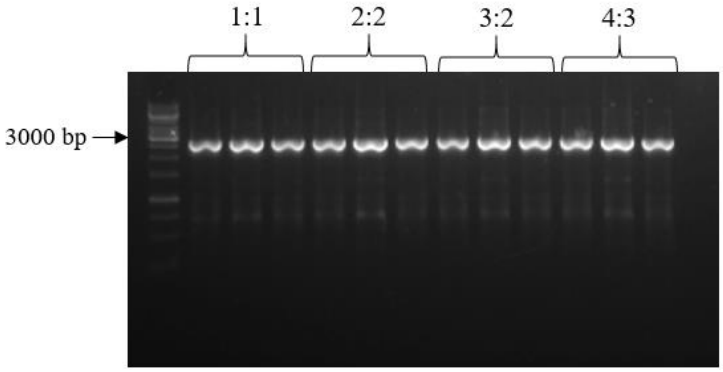


Figure 24. Amplification of luciferase-rhoptry associated protein 3' cassette. Electrophoresis of the PCR products resultant from *luc-3'rap-1* cassette amplification having different ligation reactions as template (1:1, 2:2, 3:2, 4:3) in a 0.8 % (vol/vol) agarose gel. Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific).

The *luc*-3'*rap-1* cassette obtained was then digested with *Eco*RV and *Bam*HI restriction enzymes and ligated with the 3600 bp plasmid fragment obtained above (**Figure 23A**), correspondent to the isolated vector. In parallel, another ligation experiment was conducted: the ligation of *luc*-3'*rap-1* with all the digestion products, without isolation of the vector.

Ligation reaction proportions of 1:1 and 0.5:1 (vector: fragment) were performed and transformed in *E. coli* JM109. More colonies appeared after transformation with ligation reactions containing the isolated vector and the ratio 0.5:1 was more efficient (**Figure 25**).

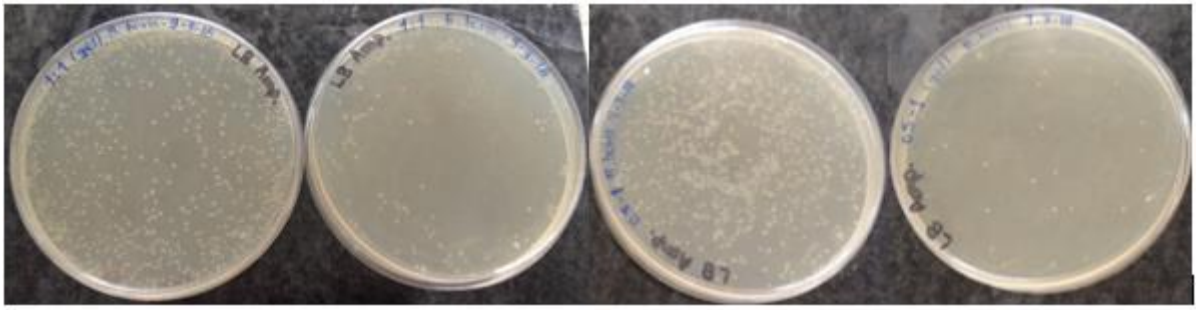


Figure 25. *Escherichia coli* JM109 colonies after transformation with different ligation reactions to originate pBbovis-luc. From left to right, ligation reaction with: isolated vector at a 1:1 (vector: fragment) ratio, non-isolated vector at a 1:1 ratio, isolated vector at 0.5:1 ratio and non-isolated vector at 0.5:1 ratio.

After one round of replication, a colony screening PCR was performed using primers targeting *luc*-3'*rap-1* cassette amplification and two colonies were shown to be positive for the presence of the fragment, one correspondent to passage of *E. coli* JM109 transformed with a ligation reaction containing the isolated vector (1:1 ratio) and the other with the non-isolated vector (0.5:1 ratio). After a second replication round of the positive clones followed by colony screening PCR, only 3 positive clones for the *luc*-3'*rap-1* fragment were obtained, of which DNA was extracted and analysed by enzymatic restriction. Cleavage with *Eco*RV and *Bam*HI produced two fragments, one with approximately 3500 bp, corresponding to the vector (3671 bp size), and a fragment between 2500 and 3000 bp that should correspond to the *luc*-3'*rap-1* cassette. Incomplete digestion of some clones was observed (clones 3a and 3b), whereas a unique fragment was produced with approximately 6000 bp correspondent to the linearized vector (**Figure 26).**

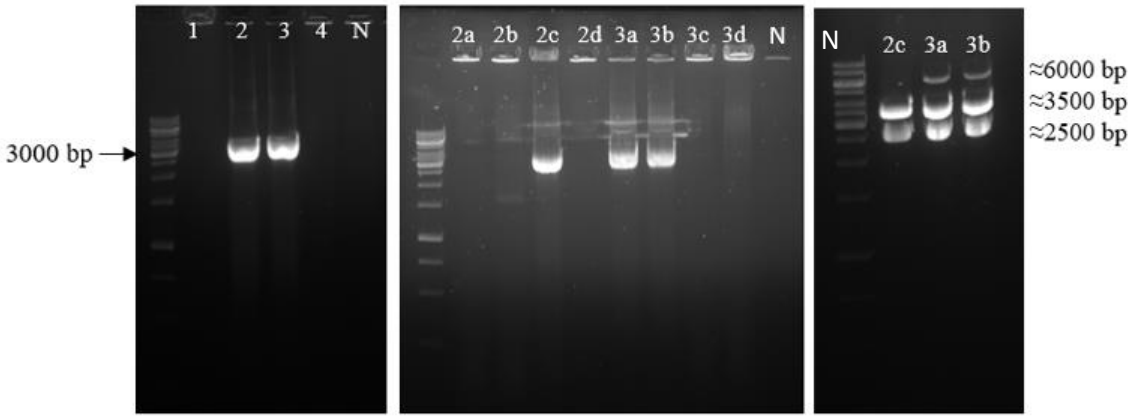


Figure 26. Colony screening PCR in transformed *Escherichia coli* JM109 with expected pBbovis-luc targeting *luc-3'rap-1* cassette amplification. From left to right: Electrophoresis of the PCR products resultant from a colony PCR to a passage from *E. coli* JM109 transformed with the following ligation reactions: isolated vector at a 1:1 (vector: fragment) ratio (1), non-isolated vector at a 1:1 ratio (2), isolated vector at 0.5:1 ratio (3) and non-isolated vector at 0.5:1 ratio (4) and to passages from the previously mentioned (2a→3d) in a 0.8 % (vol/vol) agarose gel. N: Negative control. PCR product approximate size and the was determined by comparison with GeneRuler 1 kb DNA Ladder.

Confirmation of correct fragment insertion was performed by sequencing clone 3a with primers PR-luc-seq., PF-luc-seq and M13F-pUC (-40), and after several alignments it was detected that *luc* gene sequence differed from the template sequence used to design the primer **(Appendix VII.1)**. After accessing the sequence of the *luc* gene present in pLVX-TetOne™-Puro-Luc, it was found that the sequence used to design the primers was different. Given the hybridization site of the primers designed, the *luc* gene present in pBrfp-bsd was probably truncated. Promptly, a new reverse primer was designed to appropriately amplify *luc* gene and a correct pBrfp-bsd was constructed as it follows.

Clone 3a was digested with *Bgl*II and *EcoR*V restriction enzymes and *luc* gene was excised with the same enzymes. Ligation reactions were performed with vector: fragment ratios of 1:3, 1:5 and 0.5:3 and PCR colony screening targeting *luc* gene was performed throughout two replication rounds of the positive clones. Restriction digestion of clones 6, 8, 9 and 12 was performed with *Bgl*II and *Hind*III restriction enzymes which should produce 3 fragments, one with approximately 800 bp corresponding to the promoter region, other with approximately 1600 bp correspondent to *luc* gene and a third fragment correspondent to the remaining vector (≈3600 bp). The absence of this pattern was probably related with an incomplete digestion and, PCR aiming amplification of *luc* gene was performed to the same clones, resulting in the amplification of a fragment with the expected size in clone 9 (**Figure 27**).

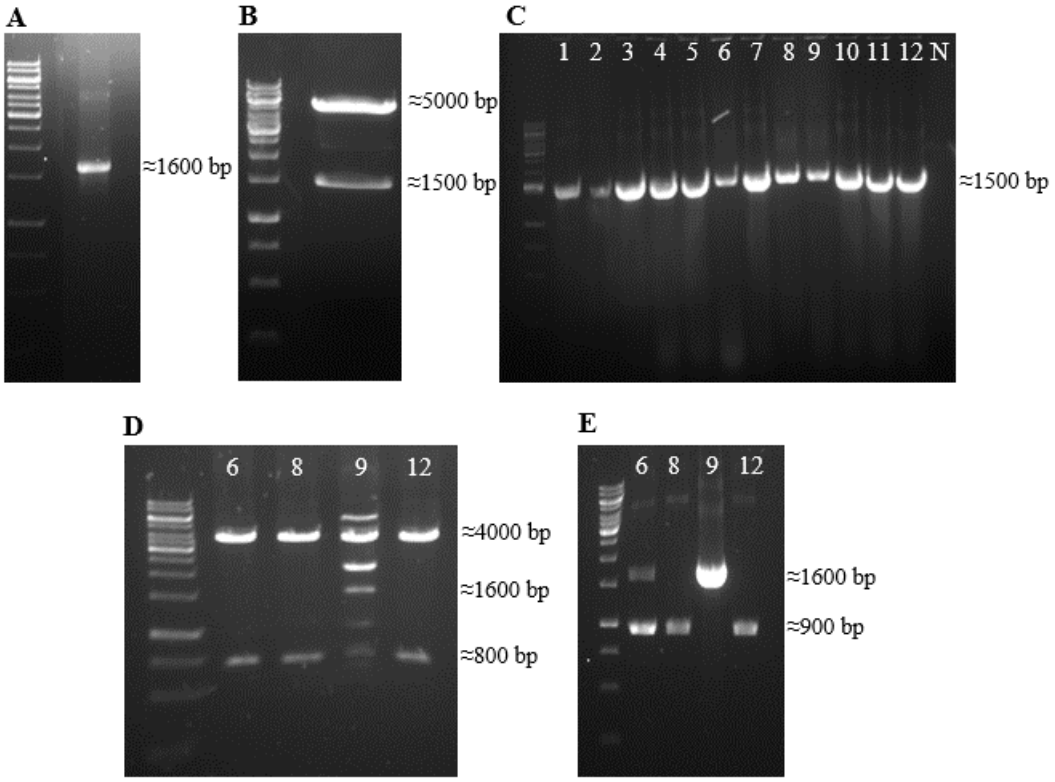


Figure 27. Development and validation of plasmid with *Babesia bovis elongation factor-1alpha* Intergenic region-B driving expression of *firefly luciferase* gene (pBbovis-luc). Electrophoresis of the **(A)** PCR product resultant of *luc* gene amplification with pLVX-TetOne™-Puro-Luc as template, **(B)** products of clone3a digestion with *Eco*RV and *Bam*HI restriction enzymes, **(C)** PCR products resultant from a colony PCR targeting *luc* gene (1→12), **(D)** restriction digestion of clones 6, 8, 9 and 12 with *Hind*III and *Bgl*II restriction enzymes and **(E)** PCR products resultant of *luc* gene amplification with clones 6, 8, 9 and 12 as template, in a 0.8 % (vol/vol) agarose gel. N: Negative control. Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder.

Correct integration of *luc* gene was validated by sequencing clone 9 (**Appendix VIII**).

4.2.2. Development of plasmid with *Babesia ovata elongation factor-1alpha* intergenic region-B driving expression of a reporter luciferase

The pBef1αIG2 was used as backbone for the development of pBovata-luc. Briefly, the pre-existing reporter Renilla Luciferase, was excised from the plasmid and replaced by *luc* gene. To achieve this, pBef1αIG2 was digested with the *Eco*RV and *Eco*RI restriction enzymes and *luc* gene was PCR amplified from pLVX-TetOne™-Puro-Luc and cleaved using the same restriction enzymes. Ligation reactions were performed with 1:3, 1:5 and 0.5:3 (vector: fragment) molar ratios, being the first more efficient. Moreover, two replication rounds were performed, and 7 selected colonies were all positive for the presence of *luc* gene (**Figure 28**). Correct integration of *luc* gene was confirmed by sequencing clone 3 with M13F-pUC (-40) (**Appendix IX**).

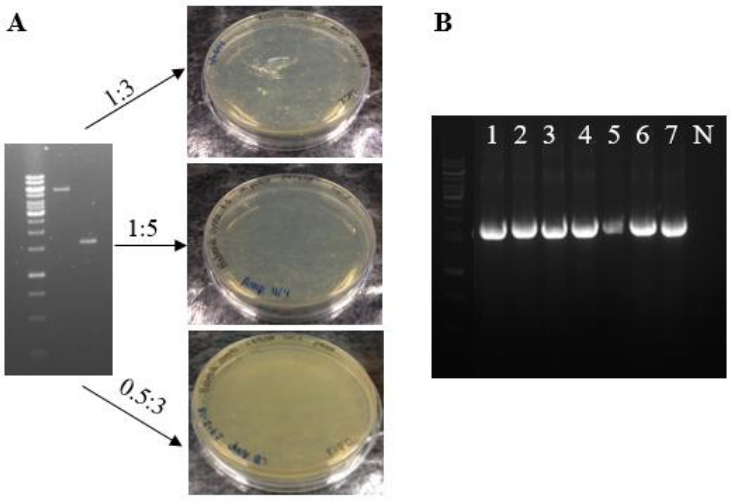


Figure 28. Schematics representing the development and validation of plasmid with *Babesia ovata elongation factor-1alpha* Intergenic region-B driving expression of *firefly luciferase (luc)* gene (pBovata-luc). **(A)** From left to right: Electrophoresis to define ligation ratios between the plasmid vector resultant from pBef1αIG2 restriction digestion with *EcoR*V and *Bam*HI restriction enzymes and the purified PCR product resultant of *luc* gene amplification from pLVX-TetOne™-Puro-Luc, digested with the same restriction enzymes in a 0.8 % (vol/vol) agarose gel. *E. coli* JM109 colonies resultant from transformation with 1:3, 1:5 and 0.5:3 (vector: fragment) ligation molar ratios are also observed. (**B**) Electrophoresis of the PCR products resultant from a colony screening PCR targeting *luc* gene performed to 7 colonies of a second replication round in a 0.8 % (vol/vol) agarose gel. N: Negative control. Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder.

4.2.3. Development of a promoterless plasmid

A promoterless plasmid, pBS-luc, was also developed to be used as negative control in the luciferase assays. Briefly, pBovata-luc was digested with *Hind*III restriction enzyme excising the 845 bp *ef1α*-B promoter sequence. After vector re-ligation, validation of promoter excision was performed by digestion with *Hind*III which resulted, as expected in the linearized pBS-luc (**Figure 29**).

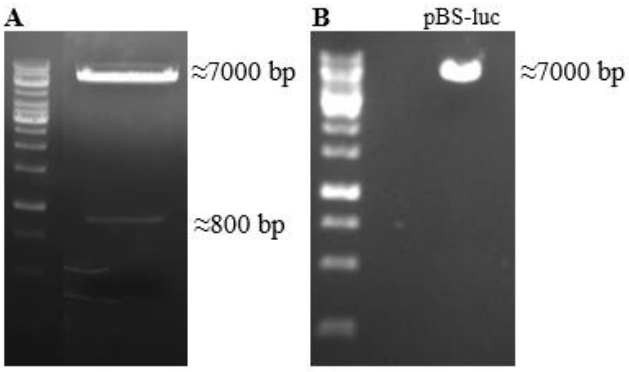


Figure 29. Development and validation of the promoterless plasmid (pBS-luc). Electrophoresis of the products resultant from restriction digestion of **(A)** pBovata-luc with *Hind*III restriction enzyme and **(B)** promoterless plasmid (pBS-luc) with *Hind*III restriction enzyme in a 0.8% (vol/vol) agarose gel . Products approximate size was determined by comparison with GeneRuler 1 kb DNA Ladder.

4.3. Preliminary transfection of *Babesia ovis* by nucleofection

4.3.1. Transfection of *Babesia ovis* by nucleofection: a comparison between two nucleofection buffers

Experimental conditions were determined for short term transfection of *B. ovis*. Until now, no pulse condition or relevant nucleofection solution has been recommended by Amaxa for nucleofection of *Babesia* spp. Therefore, the present work initiated with the selection of Amaxa nucleofector program V-024 with transfection of 20 µg of plasmid. The initial transfection experiments allowed to compare transfection outcomes between two different Amaxa nucleofector buffers named "T-cell" and "Parasite". Evaluation of luminescence activity expressed in RLU was performed for a 72h period and transfection efficiency expressed in a luc/ "genome" copies ratio was performed 24h post-transfection. Moreover, the effect of each nucleofection solution on parasite viability was evaluated 24h after transfection.

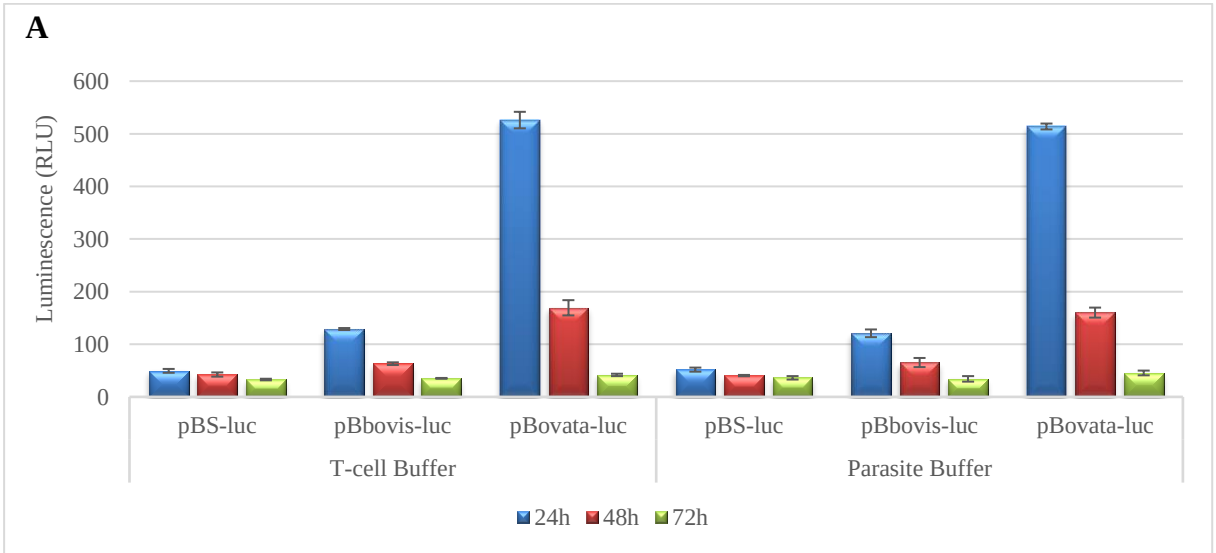
Transfection was performed with both plasmids since no information regarding the ability of heterologous promoters to promote expression in *B. ovis* was available. Initial results demonstrated that *B. ovis* transcriptional machinery had been able to recognize both promoters and drive expression of the reporter luciferase.

Assuming that an increase of transfection experiments would reflect the results herein shown, one can suggest that luminescence is equivalent for the same transfected plasmid using different transfection buffers, throughout the 72 hours. Negative controls present background luminescence (identical to the empty wells). These results are in accordance with a previous study in which *B. bovis* was transfected using two different buffers, the "Plasmodium 88A6", herein named "Parasite" and the "T-cell". The absolute luminescence values obtained then were in the range of the ones obtained for transfection with pBovata-luc and, in agreement with the present data, no statistic significant differences were found between the two buffers [92,174].

Concerning the transfection efficiency, evaluated 24h after transfection, it was possible to observe that, for all the plasmid constructs, there is a higher ratio of luc/ "genome copies" after transfection with the T-cell buffer, even though this does not correlate with the previous luminescence values (**Figure 30B)**. Transfection efficiency was calculated as the ratio between *luc* and *BoSPD* genes after extracting gDNA from the parasite culture. Since the cytoplasm contains nucleases that rapidly degrade free DNA, this assessment is precise in quantification of the amount of plasmid DNA that reached the nucleus, supporting the use of this evaluation in the present and previous transfection studies [90,95,128,175,176].

Herein, we can observe that transfection with pBovata and pBbovis translates in equal luminescence values for both transfections with T-cell and Parasite buffer, but transfection efficiency increases between 1.5 to 2 times using the T-cell buffer. The quantification of a *B. ovis* gene, *BoSPD*, does not allow to discriminate between death and live parasites [177] which will not affect our experiments if using the same transfection conditions between trials. This fact will not affect following experiments since the parasite survival post-nucleofection should be identical throughout the study. Assuming that this preliminary evaluation is correct, the differences between transfection buffers can be explained by the effect of buffers in parasite viability (**Figure 30C)**. Thus, while more plasmid DNA entries the nucleus when using the T-cell buffer, this solution also renders less viable parasites to express the reporter luciferase. The equivalent luminescence values indicate that the increase on plasmid DNA entry is inversely proportional to parasite survival. The nucleofection process has a clear detrimental effect on parasite survival (initial PPE was of approximately 6% for all the transfections carried out), however no statistically significant difference was found in the PPE obtained 24h post-transfection with different buffers (**Figure 30C)**.

Overall, transfection efficiency of pBbovis-luc is reduced in comparison to the other plasmid constructs, suggesting that the lower luciferase expression detected can also be related to a lower transfection efficiency and not only with a reduced promoter activity. More transfection experiments were performed to evaluate these promoters and are detailed in the following section. Considering that no differences were found between the studied buffers in luminescence activity and previous nucleofection studies in *B. bovis* and *B. ovata*, the human T-cell solution transfection solution was selected to further experiments [7,94,108].



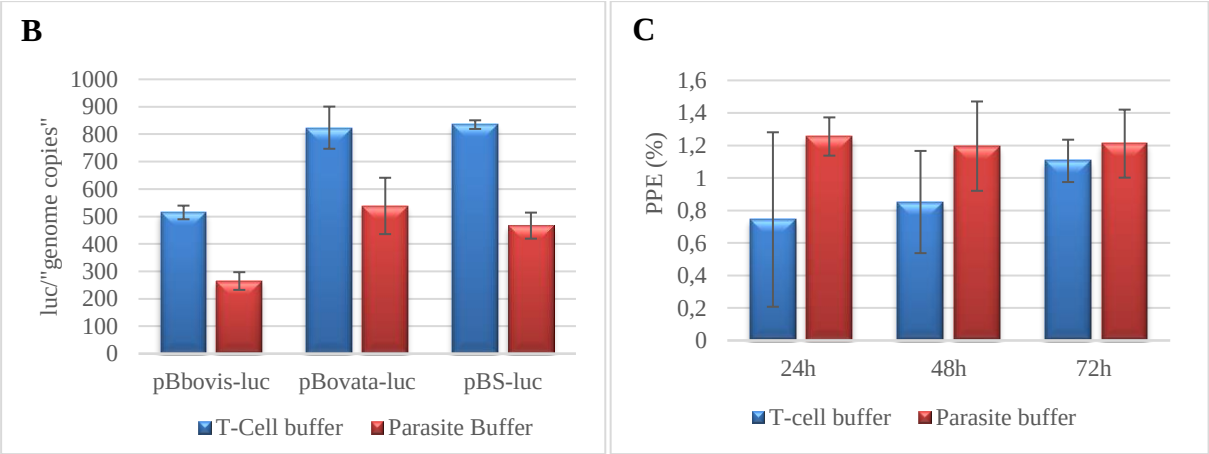


Figure 30. Transfection of *Babesia ovis* by nucleofection. (A) Comparison of luciferase expression in *B. ovis* transfected with plasmids pBbovis-luc, pBovata-luc and pBS-luc (negative control) using two different Amaxa nucleofector buffers: T-cell and Parasite. The luciferase assays were performed 24h, 48h and 72h post-transfection. RLU: Relative Luminescence Units. Data is expressed as the mean of the obtained RLUs for each transfection ±SD.(n=1) **(B)** Comparison of transfection efficiency in in *B. ovis* transfected with plasmids pBbovis-luc, pBovata-luc and pBS-luc using two different Amaxa nucleofector buffers: T-cell and Parasite 24h post-transfection. A qPCR was carried out to assess the number of plasmid DNA copies per "genome" targeting *luc* and *BoSPD* genes. The ratio of plasmid / "genome copies" was calculated assuming that *BoSPD* has one copy per genome and used has a measure of transfection efficiency. Data is expressed as the mean of luc/"genome" copies ratio obtained from three technical replicates for each transfection ±SD. (n=1) **(C)** Percentage of parasitized erythrocytes (PPE) in *B. ovis* cultures obtained 24h, 48h and 72h after nucleofection with the T-cell buffer and the Parasite buffer. Data is expressed as the mean PPE after three independent transfections ±SD. Data obtained 24h post-transfection was compared using a paired two sample *t* test and no statistically significant difference was found among means ($p<0.05$).

4.3.2. Optimal plasmid DNA amount

To determine the optimal quantity of plasmid for transfection *B. ovis* , 20 µg or 50 µg of pBbovis-luc, pBovata-luc and pBS-luc (using the previously selected T-cell buffer) were used. Transfection of more quantity of pBovata-luc correlated with an increase in the RLU absolute value **(Figure 31A)** and, interestingly, when expressing those same RLUs relatively to the amount of DNA there is also an increase of the luminescence activity *per* microgram of plasmid **(Figure 31B)**. Regarding pBbovis-luc it was also observed a slight increase on absolute luminescence activity when transfecting more quantity of plasmid DNA. However, when the data is plotted to express RLU relatively to the DNA amount a slight decrease occurs **(Figure 31A and B).** A previous study used varying amounts of plasmid DNA, between 2 µg and 200 µg, demonstrating that increasing the amount of plasmid DNA correlates with a decrease in the RLU/µg values [92]. In that study, data suggests that the increase of plasmid DNA from 20 µg the 50 µg correlates with a slight decrease in the RLU/µg value which our transfection experiment with pBbovis-luc corroborates. However, a more recent study with transfection of *B. ovata* reports that increasing the amount of plasmid DNA from 50 µg to 100µ correlates with an

increase in transfection efficiency [94]. In the conditions undertaken in the present study, higher plasmid DNA did not show to affect parasite viability since no statistically significant differences were found when comparing different plasmid amounts **(Figure 31C)**. In a previous study it was found that increasing the plasmid amount would correlate with a decrease on parasite viability however in it was only evaluated transfection with 2 µg and 200 µg, so this data cannot be compared with the presently obtained [92].

At the light of the results obtained, the following transfection experiments were conducted using 20 µg of plasmid DNA.

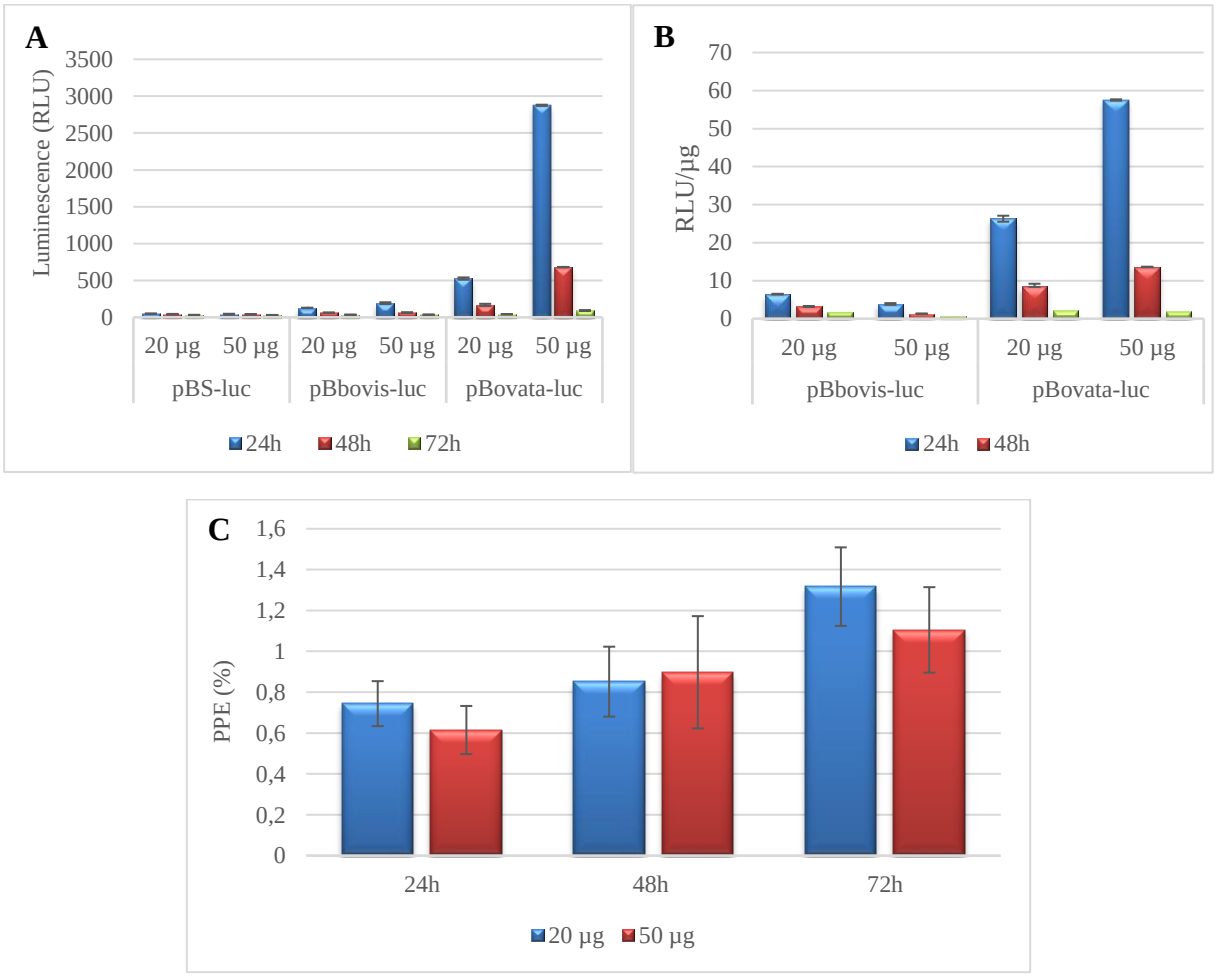


Figure 31. Transfection of *Babesia ovis* using varying amounts of plasmid DNA. (A) Comparison of luciferase activity 24 h, 48 h and 78 h post-nucleofection with varying amounts (20 µg and 50 µg) of pBbovis-luc, pBovata-luc and pBS-luc (negative control)(n=1). **(B)** Comparison of the ratio of luciferase activity relatively to the amount of DNA transfected. RLU: Relative Luminescence Units. Data is expressed as the mean of the obtained RLUs for each transfection ±SD (n=1). **(C)** Percentage of parasitized erythrocytes (PPE) in *B. ovis* cultures obtained 24h, 48h and 72h after nucleofection with 20 µg and 50 µg of plasmid DNA. Data is expressed as the mean PPE after three independent transfections ±SD. Data obtained 24h post-transfection was compared using a paired two sample *t* test and no statistically significant difference was found among means ($p<0.05$).

Noteworthy, in these initial transfection experiments a positive control was also included, the pmaxGFP^{TM} vector supplied by Amaxa biosystems. This expression vector has a CMV promoter, and until now, thought that this promoter was not recognized by apicomplexan parasites transcriptional machinery [125,126]. Herein, activity was observed, urging additional studies to clarify if *B. ovis* transcriptional machinery is indeed able to recognize CMV promoter sequence.

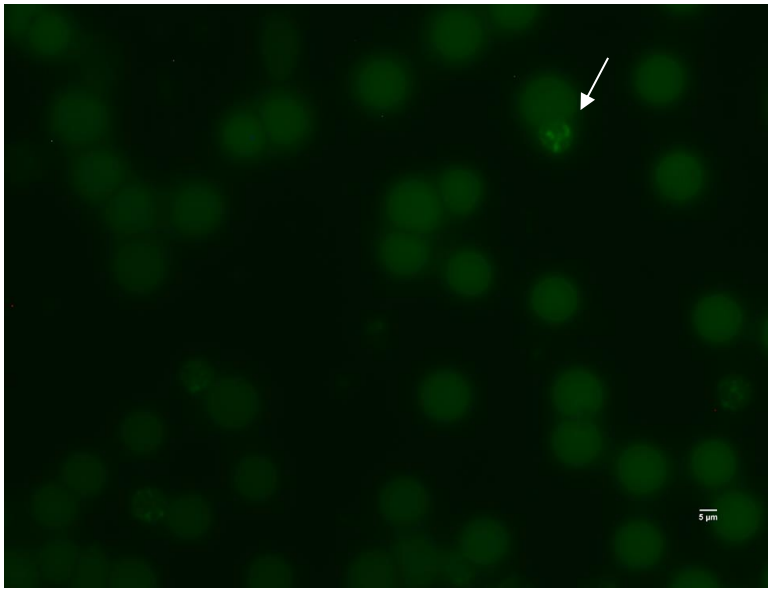


Figure 32. Fluorescence microscopy image of *Babesia ovis* expressing green fluorescent protein (GFP) after transfection with pmaxGFP^{TM} vector. Intraerythrocytic parasites were observed under a 400x original magnification of a Nikon eclipse 80i fluorescence microscope with the GFP filter. *Scale-bar:5 µm*

4.4. *Babesia bovis* and *Babesia ovata elongation factor-1alpha* intergenic region-B have heterologous promoter activity in *Babesia ovis*

The promoter activity of *B. bovis* and *B. ovata ef-1* IG-B regions were tested using luciferase assays. Both promoter regions used in the present study were previously validated concerning their ability to drive expression of a reporter gene in their respective species [94,108]. After transfection with the transient plasmid constructs developed (**Figure 33A**), luciferase expression was evaluated for a time lapse of 24h-72h post *B. ovis* nucleofection. It was observed maximum luciferase expression at 24h post-transfection with either pBbovis-luc and pBovata and, 72h post-transfection, the luminescence expression is negligible **(Figure 33B).** In a recent study, *B. bovis* was transfected with a plasmid containing *B. bigemina ef-1α* IG-B driving expression of a reporter luciferase and a similar pattern of luciferase expression was observed throughout the 72h period, with the peak of luciferase expression occurring 24h post-transfection [95]. Similarly, transient transfection of *B. gibsoni* with the homologous 5' *ef-1α* driving expression of the same reporter gene resulted in a decrease of luminescence along the

72h period [97]. In contrast, another study in *B. ovata* with a homologous promoter driving expression of a reporter luciferase shows a peak of luciferase activity 48h post-transfection an expression that is maintained until 96h [94]. These differences are probably related with the different growth rate of these different species since upon cell division the transiently transfected genetic material is lost **(Figure 34)** [85,97].

Herein, we demonstrate that both *B. ovata* and *B. bovis ef-1α* IG-B regions are recognized by *B. ovis* transcriptional machinery, being able to drive expression of a reporter luciferase. Furthermore, *B. ovata ef-1α*-B promoter region can induce expression of higher levels of luciferase indicating that *B. ovis* transcription factors have a higher binding affinity towards motifs existent in *B. ovata ef-1α* IG-B region [120]. These transcription factors can be either sequence-specific DNA-binding proteins involved in specific regulation of a certain gene or more general, related with RNA polymerase II components required for transcription initiation. However, absence of knowledge regarding *B. ovis cis*-regulatory motifs does not allow to infer which binding factors might be weighting in the presently obtained results [113,119]. Recently, it was attempted to transfect *B. ovata* with a plasmid having *B. bovis ef-1α* IG-B region, but this region was not able to induce expression of the reporter luciferase, a fact attributed to a lack of recognition by either *B. ovata* transcriptional or translational machinery [94]. Therefore, one can suggest that the sequence of *B. bovis ef-1α* IG-B does not have certain motifs necessary for transcription factor recognition by *B. ovata*, being probably quite different from the *B. ovata* homologous *ef-1α* IG-B sequence. Thus, supporting the differences observed in the luciferase expression of these promoter regions in *B. ovis.*

Another fact that can explain the differences observed in luciferase expression is transfection efficiency **(Figure 33C)**. However, no significant differences in transfection efficiencies were found between pBbovis and pBovata this last hypothesis can be precluded and vehemently suggest that *B. ovis* transcriptional factors have a stronger affinity for *B. ovata* than for the *B. bovis* ef-1a-B promoter sequence.

The present results also indicate that both *B. bovis* and *B. ovata* 3' *rap-1* terminator regions have a cross-species function in *B. ovis.* The cross-species function of this region had only been described once before with *B. bovis* 3' *rap-1* terminator region being functional in *B. gibsoni* [100]. A study that analysed *rap-1* IG regions from *B. bovis*, *B. bigemina*, *B. canis* and *B. ovis* concluded that the sequence conservation between species was relatively low with the highest sequence similarity (42% identity) being found between *B. ovis* and *B. bovis* sequences [157]. It appears that both *B. bovis* and *B. ovata rap-1* IG regions have the necessary termination signals to be functional in *B. ovis.*

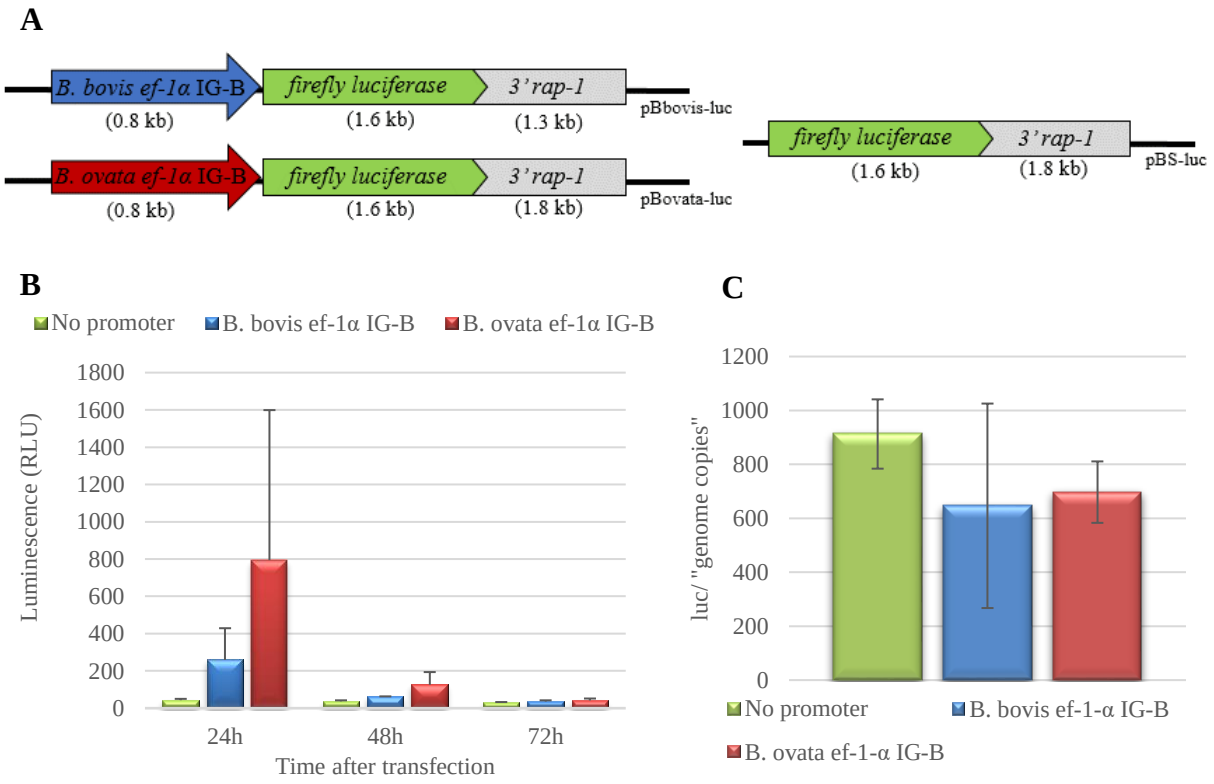


Figure 33. Schematic diagram of the plasmids used for transient transfection and evaluation of *Babesia bovis* and *Babesia ovata elongation factor-1alpha* intergenic region-B heterologous promoter activity in *Babesia ovis*. (A) Transient transfection constructs with *B. bovis* and *B. ovata ef-1α*-B promoter sequence driving expression of luciferase as a reporter were developed as well as a promoter-less plasmid to use as negative control. **(B)** Evaluation of the promoter activity of *B. bovis* and *B. ovata ef-1α* IG-B driving expression of a reporter luciferase over a time lapse of 24-72h post-transfection in B. ovis. Negative control was performed through transfection of a promoter-less plasmid. Data are expressed as the mean of the obtained RLUs of three independent transfections with each plasmid *(B. bovis ef-1α* IG-B, *B. ovata ef-1α* IG-B and promoter-less plasmid) + SD. RLU: Relative luciferase units. Data were compared using One-way ANOVA analysis, with no significant differences ($P < 0.05$) detected, ($F(2,6)=1.989$, $P=0.2174$ at 24h, $F(2,6)=1.306$, $P=0.3383$ at 48h and $F(2,6)=1.73$, $P=0.2552$ at 72h).**(C)** Evaluation of transfection efficiency of the plasmid constructs by the luc/"genome copies" ratio. A qPCR was carried out to assess the number of plasmid DNA copies per "genome" 24h post-transfection targeting luc and BoSPD genes. The ratio of plasmid /"genome copies" was calculated assuming that BoSPD has one copy per genome. No statistically significant differences among the plasmids transfected were found (One-way ANOVA Analysis ($p < 0.05$), $F(2,6)=1.053$, $P=0.4055$)

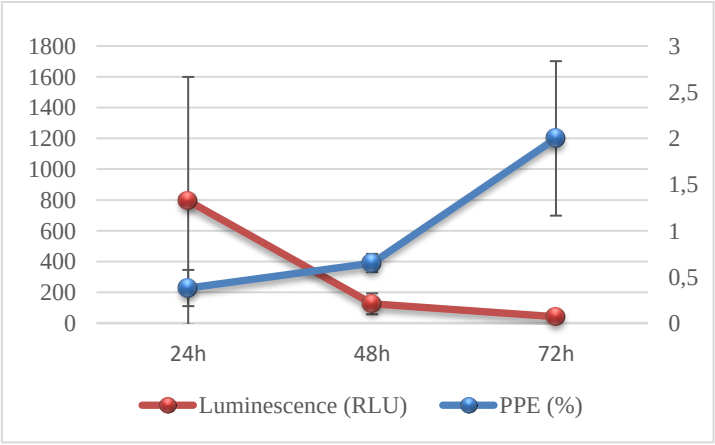


Figure 34. Time course of luciferase activity and parasitemia measured at 24h, 48h and 72h post-transfection with pBovata-luc. RLU: Relative luciferase units. PPE: Percentage of parasitized erythrocytes. Data is expressed as the mean of three independent transfections ±SD.

4.5. Growth inhibition of *B. ovis* by blasticidin-S and the effect of WR99210 in *in vitro* cultures

To determine if WR99210 could inhibit *B. ovis in vitro* growth as well as a suitable concentration for selection of transgenic parasites, *B. ovis* Israeli strain was subjected to different drug concentrations ranging from 0 to 50 nM. The initial PPE for all the culture wells was 1% and it was determined daily for a period of 72h. The results showed that after 3 days of drug selection only 17,7% of growth inhibition was achieved at the higher concentration tested and it was not possible to determine the drug IC_{50}. No significant differences between the PPE attained in the culture wells exposed to different drug concentrations and the control wells (only with DMSO, the drug vehicle) (**Figure 35**) was determined. Thus, WR9910, showed not to be an efficient *B. ovis* growth inhibitor. Interestingly, these results are contrary to what is described for other WR99210 sensitivity assays in *Babesia* spp. For *B. bovis* the IC_{50} determined was 1.0 nM and, at a concentration of 5 nM, parasite growth was completely inhibited, similar to the growth inhibitory concentrations found in *Plasmodium* parasites [7,108,178] For *B. ovata*, WR99210 completely inhibits *in vitro* growth at a nanomolar concentration (5 nM) with an IC_{50} of 0.56 ± 0.01 nM and for *B. gibsoni* the calculated IC_{50} was 1.1 nM, similar to the described for *B. bovis*, with the drug at 10 nM completely inhibiting the parasite growth [94,98].

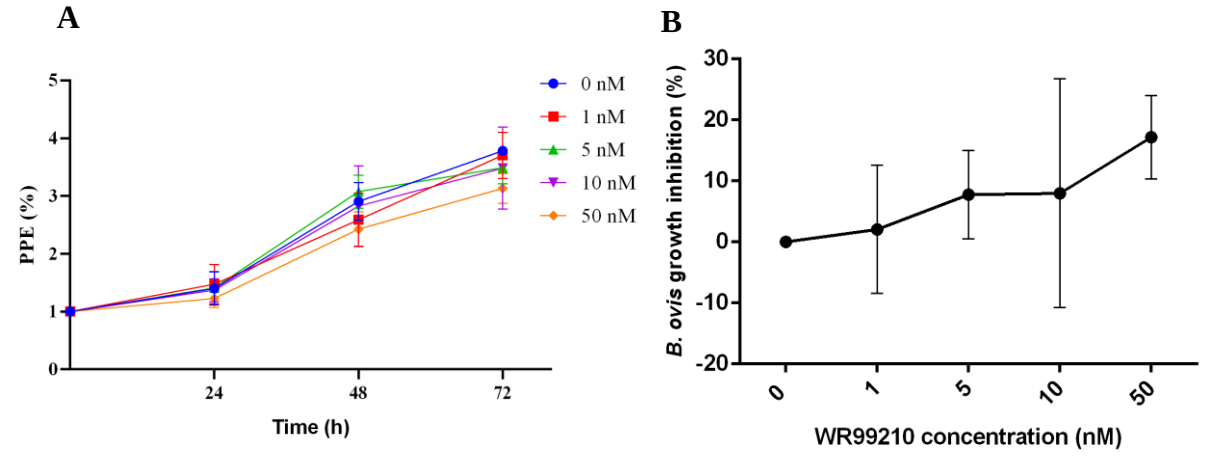


Figure 35. *In vitro* growth curve (A) and growth inhibition of *Babesia ovis* Israeli strain with WR99210 (B). Parasite cultures were subjected to different concentrations of WR99210 (0-50 nM) and the percentage of parasitized erythrocytes (PPE) was determined every 24h over a period of 72h. All data are expressed as the arithmetic means ± SD of the triplicate cultures

Reports from different research groups assign the lack of growth inhibition to the drug manufacturer. Thus, this is the most plausible explanation for WR99210 failure to inhibit *B. ovis* in vitro growth more than the achieved 17,7%. The presence of polymorphic copies of the *dhfr* gene in *B. ovis* genome that would reduce the parasite sensitivity to this antifolate drug can

also be considered. However, the non-emergence of naturally resistant parasites in previous selected transfected *Babesia* spp. does not support this hypothesis[94,98]. To consider WR99210/*dhfr* as selection system for *B. ovis,* higher drug concentrations would need to be evaluated.

Given the previous results, blasticidin-S was evaluated as an alternative antibiotic. Similarly, *B. ovis* Israeli strain was cultured in the presence of different blasticidin S concentrations, ranging from 0 μg/mL to 28.8 μg/mL for a period of 72h with PPE being monitored in a daily basis. The results show that increasing blasticidin concentration above 4.8 μg/mL correlates with a decreasing PPE at 24h and after 48h this value is lower with drug concentration above 2.4 μg/mL correlating with a decrease in PPE (**Figure 36A**). At 72h, blasticidin-S concentrations above 4.8 μg/mL result in negligible parasite growth (**Figure 36B and Appendix X.1).** To ensure that concentrations above 19.2 μg/μL inhibited completely parasite growth the cultures subjected to that concentration range were maintained for more 96 hours without the drug pressure to confirm that the parasites observed in the end of the sensitivity assay were not able to re-infect other erythrocytes. Indeed, after that period no parasites were observed.

The calculated IC_{50} found was approximately 2.3 μg/mL and concentrations in 4.8 – 28.8 μg/mL range are suggested to inhibit completely parasite growth. Interestingly, the obtained results are quite different from the ones obtained in the first sensitivity assay performed in *B. bovis,* for which the blasticidin-S calculated IC_{50} was 0.4 μg/mL and the concentration range of 0.64–1.25 μg/mL resulted in negligible parasite growth [93]. In a recent study, the calculated IC_{50} for *B. bovis* was 0.8 μg/mL and concentrations between 3 μg/mL and 9 μg/mL completely inhibited parasite growth. In the same study, the IC_{50} for *B. bigemina* was 3 μg/mL and drug concentrations between 8 μg/mL and 14 μg/mL would correlate with complete *in vitro* growth inhibition, results more similar to the found for *B. ovis* in the present study [96].

Overall, the present results demonstrate the suitability of using blasticidin-S/*bsd* selection system in the development of a stable transfection system for *B. ovis.* Moreover, the resistant determinant, the *bsd* gene is encoded by a small ORF (399 bp) which further enhances the probability of an efficient transfection and allows the cloning of larger DNA fragments without compromising transfection efficiency [142].

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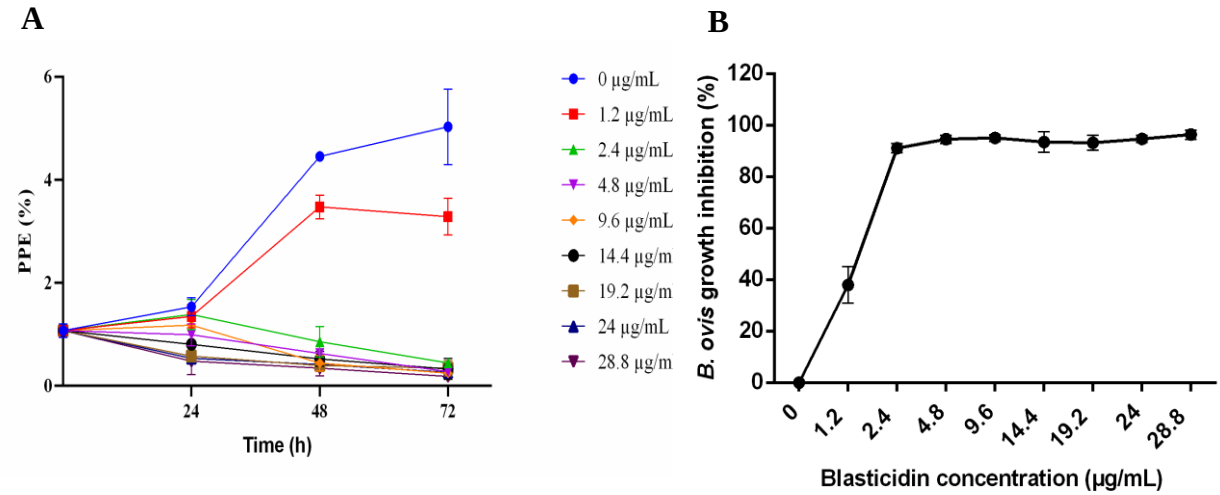

Figure 36. *In vitro* growth curve (A) and growth inhibition of *Babesia ovis* Israeli strain with blasticidin-S (B). Parasite cultures were subjected to different concentrations of blasticidin (0-28.8 µg/mL) and the percentage of parasitized erythrocytes (PPE) was determined every 24h over a period of 72h. All data are expressed as the arithmetic means ± SD of the triplicate cultures

4.6. Construction and preliminary validation of a recombinant plasmid expressing an RFP-BSD fusion protein

The mechanisms for integration of exogenous DNA and the occurrence of legitimate recombination events involving sequences in the transfection plasmid (promoter, terminator and flanking regions) were studied in *B. bovis.* The pattern of integration consisted mainly in the disruption of the *ef-1α-B* gene, and as so the design of the transfection plasmid included 5' and 3' *ef-1α-B* as flanking regions. However, another observable pattern of integration included part of the *ef-1α-B* promoter sequence in the recombination event. This led to endorse the use of heterologous promoter and terminator regions in future transfection plasmids [8]. Thus, results obtained so far, supported the use of *B. ovata ef-1a-B* promoter region to drive expression of an RFP-BSD fusion protein in a future stable transfection system aiming genome integration by disruption of *B. ovis ef-1a-B* gene.

The pBrfp-bsd was used as backbone for the development of pBovata-rfp-bsd **(Table 2).** Briefly, the pre-existing *B. bovis ef-1α* IG-B region was excised from the plasmid and replaced by *B. ovata ef-1α* IG-B region. To achieve this, the plasmid was cleaved with *Sal*I and *Hind*III and isolated from the gel. In parallel, *B. ovata ef-1α* IG-B region was amplified by PCR from pBef1αIG2, purified and cleaved with the same restriction enzymes **(Figure 37A)**. Ligation was performed and *E. coli* JM109 colonies appeared after transformation with the ligation reaction. Validation of the presence of fragment was performed by colony screening PCR targeting the *B. ovata ef-1α* IG-B and, throughout two replication rounds performed to 7

selected colonies, all the clones were positive for the presence of *B. ovata ef-1α* IG-B region (**Figure 37B**).

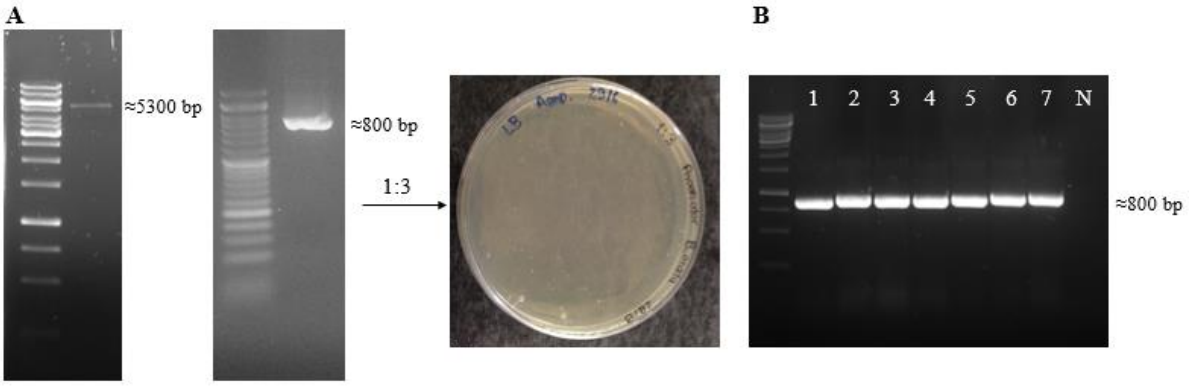

Figure 37. Schematics of the development of a plasmid with *Babesia ovata elongation factor-1alpha* Intergenic region-B region driving expression of red fluorescent-blasticidin-S deaminase fusion protein. (A) From left to right: Electrophoresis of pBrfp-bsd restriction of pBrfp-bsd with *Sal*I and *Hind*III in a 0.8 % (vol/vol) agarose gel and of the purified PCR product resultant of *B. ovata ef-1α* IG-B amplification digested with the same restriction enzymes in a 1.2 % (vol/vol) agarose gel. Ampicillin resistant clones obtained after transformation of *E. coli* JM109 . (**B**) Colony screening PCR targeting *B. ovata ef-1α* IG-B from 7 colonies of a second replication round of the primary colonies after transformation. N: PCR negative control. Products approximate size was determined by comparison with (A) GeneRuler 1 kb DNA Ladder and NZYDNA Ladder VI or (B) GeneRuler 1 kb DNA Ladder.

The RFP-BSD fusion protein functionality has been previously validated in transfections experiment in *B. bovis* [108,179]. Regarding the ability of *B. ovata ef-1α* IG-B region to drive expression of this fusion protein in *B. ovis* it was confirmed by the observed chemiluminescence as shown in the previous section **(Figure 33).** Nonetheless, transient transfection of *B. ovis* with pBovata-rfp-bsd was carried out to confirm expression of the fusion protein and, fluorescence microscopy revealed the presence of *B. ovis* transiently expressing the RFP-BSD fusion protein **(Figure 38)**.

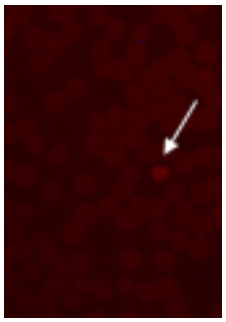

Figure 38. Fluorescent microscopy image of *Babesia ovis* transiently expressing the red fluorescent protein (RFP). Intraerythrocytic parasites were observed under a 100x original magnification of a Nikon eclipse 80i fluorescence microscope with the RFP filter.

5. Conclusions and Future Perspectives

B. ovis was successfully transfected for the first time with the establishment of a baseline protocol for future transfections of this parasite. The constructs with *ef-1α* IG-B from *B. bovis* and *B. ovata* driving expression of a reporter luciferase were developed. Both promoter regions were recognized by *B. ovis* transcriptional factors although the *ef-1α-B* promoter region from *B. ovata* showed to have a stronger affinity . The present work sustains previous reports of interspecies promoter activity between *Babesia* spp. Regrettably, given the absence of *B. ovis ef-1α* IG-B region, the interchangeable cross-species activity of this promoter region could not be explored.

B. ovis ef-1α gene was revealed to have high nucleotide sequence homology so the design of a transfection plasmid including 5' and 3' *ef-1α-B* as flanking regions is potentially a platform to stably transfect other *Babesia* spp. The establishment of this stable transfection platform common to several species appears to be feasible for at least *B. ovis* and *B. ovata.*

Currently, the development of a stable transfection system in ongoing. This will allow to have a *B. ovis* lineage stably expressing RFP, an essential tool to understand *B. ovis* and related *Babesia* spp. life cycle events in the tick vector tissues. Nevertheless, with the establishment of such system, new avenues related with functional gene characterization through gene knockout experiments and novel vaccine development are also open. For the latter and considering that *Theileria ovis* and *B. ovis* co-infection is common, an attenuated *B. ovis* stably expressing a *T. ovis* antigen could be a vaccine delivery platform enabling host immunity against both pathogens.

Advancing in the development of these transfection methods could allow using them to study key motifs essential for promoter activity. Such studies would result in the development of more efficient stable constructs, refining all the applications of this genetic manipulation method.

The development of both transient and stable transfection systems can be simplified with the release of the full genome sequence of *B. ovis* Israeli strain. The accessibility of such genomic sequences can clarify functions of proteins implicated in virulence and identify more candidates for vaccine development which can be accelerated with the application of genetic tools.

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7. Appendix

Appendix I

Appendix I.1: ***Babesia ovis actin*** **partial coding sequence obtained in the present study.**

GGAAAGAACCTATGTTGGAGATGAGCACAGGCTAAGCGTGGTATCTTGACCCTTAAATACCCAATCGA
GCATGGTATTGTCACCAACTGGGAGGATATGGAGAAAATCTGGCATCACACCTTCTACAATGAGCTTC
GTATTGCCCCTGAGGAACACCCAGTGCTGTTGACCGAAGCTCCCATGAACCCCAAGGCCAACCGTGAA
AAGATGACTACCATTATGTTCGAAACCCACAACGTGCCAGCTATGTATGTTGCTATCCAAGCCGTGCT
TTCGCTGTACTCGTCTGGTCGTACCACTGGTATTGTGCTCGACTCTGGTGATGGTGTCACCCACACCG
TCCCTATCTACGAGGGTTACGCCCTTCCCCATGCCATTATGCGTATTGACCTTGCCGGTCGTGACCTT
ACTGAGTTCATGCAAAAGATCTTGGCTGAACGTGGTTTCACCTTCACCACCACTGCCGAAAAGGAAAT
TGTGCGTGACATCAAGGAGAAATTGTGTTACATTGCACTCGACTTCGAAGAGGAAATGAACTCTTCTG
CCTCATCAAGTGAAATCGAAAAATCTTACGAACTGCCTGATGGAAACATCATCACTGTCGGAAACGAG
CGTTTCAGGTGCCCTGAGGTGCTCTTCCAACCAAGCTTCATTGGCCAAGAGTCTGCCGGTATCCACAC
CACTACCTTCAACTCAATTGCCCGTTGTGATGTCGACATCCGCAAGGACCTTTACGCAAATGTCGTCC
TTTCTGGTGGTACAACCATGTACGAGGGTATAGGCCAGCGTATGACCAAGGAGCTTAACGCTCTTGTA
CCCAGCACCATGAAGATCAAGGTCGTTGCCCCTCCTGAGCGCAAGTACTCTGTATGGATTGGAGGATC
TATTCTTTCTTCGTTGTCCACATTCCAGCAGATGTGGATCACTAAGGAGGAGTTCGACGAGTCTGACC

Appendix I.2: **Alignment between the sequence obtained in the present study and *Babesia ovis*** **actin coding sequence retrieved from *B. ovis*** **Israeli strain draft genome** (Bo_actin_CDS (g767) 1496356…1497486).

```
Bo.actin.partial      ------------------------------------------------------------    0
Bo.actin.CDS          ATGTCGGACGAAGAGGCTACCGCTTTGGTCATTGACAATGGATCCGGTAATGTCAAGGCC    60

Bo.actin.partial      ------------------------------------------------------------    0
Bo.actin.CDS          GGTGTTGCTGGAGACGATGCTCCCCGCTGCGTTTTCCCCAGCATTGTAGGTCGCCCAAAG    120

Bo.actin.partial      ------------------------------GGAAAGAACCTATGTTGGAGATGAGCACAG    30
Bo.actin.CDS          AACCCAGCCCTTATGGTTGGTATGGACGAAAAGGACACCTATGTTGGAGATGAAGCACAG    180
                                                   * * *     * *      *******

Bo.actin.partial      GCTAAGCGTGGTATCTTGACCCTTAAATACCCAATCGAGCATGGTATTGTCACCAACTGG    90
Bo.actin.CDS          GCTAAGCGTGGTATCTTGACCCTTAAATACCCAATCGAGCATGGTATTGTCACCAACTGG    240
                      ************************************************************

Bo.actin.partial      GAGGATATGGAGAAAATCTGGCATCACACCTTCTACAATGAGCTTCGTATTGCCCCTGAG    150
Bo.actin.CDS          GAGGATATGGAGAAAATCTGGCATCACACCTTCTACAATGAGCTTCGTATTGCCCCTGAG    300
                      ************************************************************

Bo.actin.partial      GAACACCCAGTGCTGTTGACCGAAGCTCCCATGAACCCCAAGGCCAACCGTGAAAAGATG    210
Bo.actin.CDS          GAACACCCAGTGCTGTTGACCGAAGCTCCCATGAACCCCAAGGCCAACCGTGAAAAGATG    360
                      ************************************************************

Bo.actin.partial      ACTACCATTATGTTCGAAACCCACAACGTGCCAGCTATGTATGTTGCTATCCAAGCCGTG    270
Bo.actin.CDS          ACTACCATTATGTTCGAAACCCACAACGTGCCAGCTATGTATGTTGCTATCCAAGCCGTG    420
                      ************************************************************

Bo.actin.partial      CTTTCGCTGTACTCGTCTGGTCGTACCACTGGTATTGTGCTCGACTCTGGTGATGGTGTC    330
Bo.actin.CDS          CTTTCGCTGTACTCGTCTGGTCGTACCACTGGTATTGTGCTCGACTCTGGTGATGGTGTC    480
                      ************************************************************

Bo.actin.partial      ACCCACACCGTCCCTATCTACGAGGGTTACGCCCTTCCCCATGCCATTATGCGTATTGAC    390
Bo.actin.CDS          ACCCACACCGTCCCTATCTACGAGGGTTACGCCCTTCCCCATGCCATTATGCGTATTGAC    540
                      ************************************************************
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Bo.actin.partial      CTTGCCGGTCGTGACCTTACTGAGTTCATGCAAAAGATCTTGGCTGAACGTGGTTTCACC    450
Bo.actin.CDS          CTTGCCGGTCGTGACCTTACTGAGTTCATGCAAAAGATCTTGGCTGAACGTGGTTTCACC    600
                      ************************************************************

Bo.actin.partial      TTCACCACCACTGCCGAAAAGGAAATTGTGCGTGACATCAAGGAGAAATTGTGTTACATT    510
Bo.actin.CDS          TTCACCACCACTGCCGAAAAGGAAATTGTGCGTGACATCAAGGAGAAATTGTGTTACATT    660
                      ************************************************************

Bo.actin.partial      GCACTCGACTTCGAAGAGGAAATGAACTCTTCTGCCTCATCAAGTGAAATCGAAAAATCT    570
Bo.actin.CDS          GCACTCGACTTCGAAGAGGAAATGAACTCTTCTGCCTCATCAAGTGAAATCGAAAAATCT    720
                      ************************************************************

Bo.actin.partial      TACGAACTGCCTGATGGAAACATCATCACTGTCGGAAACGAGCGTTTCAGGTGCCCTGAG    630
Bo.actin.CDS          TACGAACTGCCTGATGGAAACATCATCACTGTCGGAAACGAGCGTTTCAGGTGCCCTGAG    780
                      ************************************************************

Bo.actin.partial      GTGCTCTTCCAACCAAGCTTCATTGGCCAAGAGTCTGCCGGTATCCACACCACTACCTTC    690
Bo.actin.CDS          GTGCTCTTCCAACCAAGCTTCATTGGCCAAGAGTCTGCCGGTATCCACACCACTACCTTC    840
                      ************************************************************

Bo.actin.partial      AACTCAATTGCCCGTTGTGATGTCGACATCCGCAAGGACCTTTACGCAAATGTCGTCCTT    750
Bo.actin.CDS          AACTCAATTGCCCGTTGTGATGTCGACATCCGCAAGGACCTTTACGCAAATGTCGTCCTT    900
                      ************************************************************

Bo.actin.partial      TCTGGTGGTACAACCATGTACGAGGGTATAGGCCAGCGTATGACCAAGGAGCTTAACGCT    810
Bo.actin.CDS          TCTGGTGGTACAACCATGTACGAGGGTATAGGCCAGCGTATGACCAAGGAGCTTAACGCT    960
                      ************************************************************

Bo.actin.partial      CTTGTACCCAGCACCATGAAGATCAAGGTCGTTGCCCCTCCTGAGCGCAAGTACTCTGTA    870
Bo.actin.CDS          CTTGTACCCAGCACCATGAAGATCAAGGTCGTTGCCCCTCCTGAGCGCAAGTACTCTGTA    1020
                      ************************************************************

Bo.actin.partial      TGGATTGGAGGATCTATTCTTTCTTCGTTGTCCACATTCCAGCAGATGTGGATCACTAAG    930
Bo.actin.CDS          TGGATTGGAGGATCTATTCTTTCTTCGTTGTCCACATTCCAGCAGATGTGGATCACTAAG    1080
                      ************************************************************

Bo.actin.partial      GAGGAGTTCGACGAGTCTGACC------------------------------    952
Bo.actin.CDS          GAGGAGTTCGACGAGTCAGGACCAAACATTGTCCACAGAAAATGCTTCTAA    1131
                      ***************** *  *
```

Appendix I.3: ***Babesia ovis actin* 5' flanking region retrieved from *B. ovis* Israeli strain draft genome.**

CACAAGTAATGTTAGTTATAATGTATGAACGAATATAATTTTTGTGCCATGAGATAATGTATAATATT
TTTTACAGCAGAAAACGTTGCGTGATGAAGGGGAAGTACACATCTGGCATATGATTACCAGTACTACT
GAAGCATACACGAGACAAATGGAGGCTAGTTACGTAAACGCGGGGACCGTTGTGAATTTCGAAAGCAT
CAATCGCACTATAGAGCAAAGGTTAAATAAAGGTAATCTAGCCAATATGAGCGATGTTTGTGAATATG
ATCGTGACTCATATATTGGTATGGTTTACAAGGATGTATCTACCATGATCTGCGAGTAAGGTGACTTC
AAGATGCATTATGTAACTGGTAATAGTCACTTGTAACGGGCACCACCTAAGCACAGGGCAGTACAAAC
AGACGAGGTTAAGCTTGAATCGAAAAATATTGATATAAGCATCGGCAGACCAATGTGTTGGAGTTAAC
AGCTGTGTTAGCGACGCGTACTCCATAGCAAGGCACCGCTGCTGCCGCAGGAGATCGAAGGTCGCCCC
ACGGGCATTGATGTTTCCTCGATCCCACAAGCACAACTGTGTCGTAGTTCATACACTCTTTTTGGAAC
ATAGCTAAACGATTCCTATTGAACTTTTACTTTTGCGTAGTCCCTTAGAGGTAAGTGGTACGACAAAT
CATGGGCCTGCACAGTAGAAGCACATGTGTATATCCAAAGTGCTATGGCCTGCATCATCTTGCGCATT
TCAAAATTGTGGAGATGGTACAAAATTATTGTCAACATGCCCATTGAAATGCACTGGATGCATTAGTT
GATGTGCAAGCTCCGTGATTTTAATCTTTAGTTACATGAAACTACGTGCGACGCGATCCCCAAGGATG
CGCGACACCGGCATGATCGAATCAACGCGAGCACTGAATTCTCTGTCACGTGTTCATGTATATTCCAA
ATTTTATTAGCATCTCTCATACTTGCTCAGAAATAGTAATTCGAAAAA

Appendix I.4: **Babesiidae annotated sequence producing significant alignments with the *B. ovis actin* 5' flanking region sequence.**

Description	Maximum Score	Total Score	Percent Query Coverage	Expect Value	Maximum Percent Identity	GenBank Accession Number
Babesia bovis strain Texas *actin* gene, promoter region	116	116	40%	1^{-23}	68%	MF598086.1

Appendix II

Appendix II.1: *Babesia ovis* sequencing result of the 1500 bp amplicon obtained with the sequence similarity approach.

TCCACTTTACGTCGCTGACTAGTGAACTTTTTACAAATAAATCATTATTGCACTATACCAAGTTTGCG
TTTGCAGTTATCTGACATGACATCTAGCTCCTGCTTCGTGAATTCCAGGAGGTTCTCTGGCACGAAGA
CATCAGTGTTGGGGTTGTGCAGTATATCATTGAAGAACTCGTGACTGAGGGCTTCAAGTGGTTGGACA
CGTTCCTTTGGATCGTATCGTAAAAATTGTGCTACAAAGTCGATAGCGACTGGGGGTGTATTTTTGGG
GAACACTTTGGAGAGATCCGCTGGTCTGATATTTGGGAATGTGACATTTTGATAGTTGGGATGCATGG
CGTACATCTGTTCGATTGTTGGTGTCCCCAATACCTGTATGATCTTGACGAGCTGATCAATTGATGTG
TCTCCGGCGAACATGGGTTTACCCATTAGTAGCTCTCCGATGACACACCCAACGGACCATATATCTAT
GGCAGTGGTATATTCCGTTGCTCCAAGCATGAGCTCAGGAGCCCTGTAAAATCTTGAACAAATGTATG
CAACGCTCATTTCACCGGCAAGCAATTTCTTGGCTGACCCAAAGTCGCAAAGTTTGanaa

ACAACAAGCATAGCAACATCGGTTACGCCACAAGCAAGACGTACTTGGACGTTAAGGGTATAAATAAA
GGTGTCAAGAATGCTCAAACCACAAGTGTGATAGCTCTGAATGCGCAAAATACAACGATGCCCGGAGG
AAATACGCATAGCACAAAGGCGTCCACCGCGGACACACAGAGCACAAAGGGAACTGCCGCAGATACGC
AGAACTCGGGGATGACTAACTCGGACAATGGCACATATCTAAATGTGGAATTTAATAATGAAAGTCTG
ATGATAGCTTACTCCAAGCCACCGGGGACAACGACGTTGCGACCTCCCGAACGCCAAGACGTAGTTCA
GGCGCAAAGTCGCCATCCACATGCCCTTCAGGGCAATTCACAAGGAGGATCTAATGTCAAAGTAGCTG
AAGGGACACAAGATATGAATACGTCGGTTGGTGGGAACCAGCCTGCACAACTGCGGGGCGCAGACACA
CAAACAAGCACCGATAGCAAATACCAAGTCCAACGAATGATAGGTAATGGGTCATTCGGAGTTGTGCA
TGAAGCAGTTCATGTGGAAACCGGTAGGCGCTTTGCCATAAAGAAGGTTCTACAGGTATTGTTTTTGA
AATGTTTGTATCATAGATATCCAGGATCCACGTTATAAAAATCGGGGAACTCAGCATCATGctggGAA
CTCTCTCACCCAAATATtGnATATATGTTTGaCC

Appendix III

Appendix III.1: *Babesia ovis elongation factor 1 α* partial coding sequence obtained in the present study.

TTCAAGTACGGCCTGGGTTTTGGACAAGCTGAAGAGTGAGCGTGAACGTGGTATCACCATCGATATCA
CCCTGTGGAAGTTCGAAACCTCTAAGTACTACTACACTGTCATTGACGCCCCTGGTCACCGTGACTTC
ATCAAGAACATGATTACGGGTACCTCCCAGGCCGATGTGGCTATGCTTGTGGTGCCAGCTGAGGCTGG
TGGTTTCGAGGCTGCCTTCTCCAAGGAAGGTCAGACCCGTGAGCACGCTCTTTTGGCCTTCACCCTCG
GTGTCAGGCAGATTATTTGCGCCATCAACAAGATGGACAAGTGCGACTACAAGGAGGACCGTTACAGT
GAAATCCAGAAGGAAGTCCAGGGATACCTCAAGAAGGTCGGTTACAACACCGATAAGGTTCCCTTCGT
TGCCATCTCCGGTTTCATGGGTGACAACATGGTTGAGCGCTCCACCAACATGCCATGGTACAAGGGCA
AGACCTTGGTCGAGGCCCTCGACATGATGGAGCCCCCAAAGAGGCCCGTTGACAAGCCACTTCGTCTT
CCCATCCAGGGTGTCTACAAGATCGGTGGTATCGGTACCGTGCCCGTCGGTCGTGTCGAGACTGGTCA
GCTCAAGGCTGGTATGGTCCTCACCTTCGCCCCCAACCCCATCACCACCGAGTGCAAATCTGTTGAAA
TGCACCACGAAGTCGTTGAGGTTGCCTACCCTGGTGACAACGTCGGTTTCAACGTAAAGAACGTGTCT
ACCTCTGACATCCGCAGTGGTCACGTCGCCTCCGACTCCAAGAACGACCCCGCCAAGGCCGCTGTGTC
CTTCAGCGCCCAGGTCATTGTGCTTAACCACCCTGGTAACATCAAGGCTGGTTACACCCCCGTTGTTG
ACTGTCACACTGCCCACGTTTCCTGCAAGTTCGACGAGATCACCTGCCGTATGGAC

Appendix III.2: Alignment between the sequence obtained in the present study and *Babesia ovis elongation factor 1α* coding sequence retrieved from *B. ovis* Israeli strain draft genome (Bo_EFG_CDS (g3736) 749836…7499712).

```
Bo.ef.partial      ------------------------------------------------------------       0
Bo.ef.CDS          ATGCCGAAGGAGAAGACCCACATTAACTTGGTCGTTATCGGCCACGTCGACAGTGGCAAG       60

Bo.ef.partial      ------------------------------------------------------------       0
Bo.ef.CDS          TCGACCACCACTGGTCACTTGATCTACAAGCTTGGTGGTATTGACAAGCGTACCATCGAG       120

Bo.ef.partial      ----------------------------------------TTCAAGTACGGCCTGGGTTTTG       22
Bo.ef.CDS          AAGTTCGAGAAGGAATCCACCGACATGGGTAAGGGTTCGTTCAAGTACGCCTGGGTCTTG       180
                                                     *  *     ******** ***

Bo.ef.partial      GACAAGCTGAAGAGTGAGCGTGAACGTGGTATCACCATCGATATCACCCTGTGGAAGTTC       82
Bo.ef.CDS          GACAAGCTGAAGAGTGAGCGTGAACGTGGTATCACCATCGATATCACCCTGTGGAAGTTC       240
                   ************************************************************

Bo.ef.partial      GAAACCTCTAAGTACTACTACACTGTCATTGACGCCCCTGGTCACCGTGACTTCATCAAG       142
Bo.ef.CDS          GAAACCTCTAAGTACTACTACACTGTCATTGACGCCCCTGGTCACCGTGACTTCATCAAG       300
                   ************************************************************

Bo.ef.partial      AACATGATTACGGGTACCTCCCAGGCCGATGTGGCTATGCTTGTGGTGCCAGCTGAGGCT       202
Bo.ef.CDS          AACATGATTACGGGTACCTCCCAGGCCGATGTGGCTATGCTTGTGGTGCCAGCTGAGGCT       360
                   ************************************************************

Bo.ef.partial      GGTGGTTTCGAGGCTGCCTTCTCCAAGGAAGGTCAGACCCGTGAGCACGCTCTTTTGGCC       262
Bo.ef.CDS          GGTGGTTTCGAGGCTGCCTTCTCCAAGGAAGGTCAGACCCGTGAGCACGCTCTTTTGGCC       420
                   ************************************************************

Bo.ef.partial      TTCACCCTCGGTGTCAGGCAGATTATTTGCGCCATCAACAAGATGGACAAGTGCGACTAC       322
Bo.ef.CDS          TTCACCCTCGGTGTCAGGCAGATTATTTGCGCCATCAACAAGATGGACAAGTGCGACTAC       480
                   ************************************************************
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Bo.ef.partial      AAGGAGGACCGTTACAGTGAAATCCAGAAGGAAGTCCAGGGATACCTCAAGAAGGTCGGT       382
Bo.ef.CDS          AAGGAGGACCGTTACAGTGAAATCCAGAAGGAAGTCCAGGGATACCTCAAGAAGGTCGGT       540
                   ************************************************************

Bo.ef.partial      TACAACACCGATAAGGTTCCCTTCGTTGCCATCTCCGGTTTCATGGGTGACAACATGGTT       442
Bo.ef.CDS          TACAACACCGATAAGGTTCCCTTCGTTGCCATCTCCGGTTTCATGGGTGACAACATGGTT       600
                   ************************************************************

Bo.ef.partial      GAGCGCTCCACCAACATGCCATGGTACAAGGGCAAGACCTTGGTCGAGGCCCTCGACATG       502
Bo.ef.CDS          GAGCGCTCCACCAACATGCCATGGTACAAGGGCAAGACCTTGGTCGAGGCCCTCGACATG       660
                   ************************************************************

Bo.ef.partial      ATGGAGCCCCCAAAGAGGCCCGTTGACAAGCCACTTCGTCTTCCCATCCAGGGTGTCTAC       562
Bo.ef.CDS          ATGGAGCCCCCAAAGAGGCCCGTTGACAAGCCACTTCGTCTTCCCATCCAGGGTGTCTAC       720
                   ************************************************************

Bo.ef.partial      AAGATCGGTGGTATCGGTACCGTGCCCGTCGGTCGTGTCGAGACTGGTCAGCTCAAGGCT       622
Bo.ef.CDS          AAGATCGGTGGTATCGGTACCGTGCCCGTCGGTCGTGTCGAGACTGGTCAGCTCAAGGCT       780
                   ************************************************************

Bo.ef.partial      GGTATGGTCCTCACCTTCGCCCCCAACCCCATCACCACCGAGTGCAAATCTGTTGAAATG       682
Bo.ef.CDS          GGTATGGTCCTCACCTTCGCCCCCAACCCCATCACCACCGAGTGCAAATCTGTTGAAATG       840
                   ************************************************************

Bo.ef.partial      CACCACGAAGTCGTTGAGGTTGCCTACCCTGGTGACAACGTCGGTTTCAACGTAAAGAAC       742
Bo.ef.CDS          CACCACGAAGTCGTTGAGGTTGCCTACCCTGGTGACAACGTCGGTTTCAACGTAAAGAAC       900
                   ************************************************************

Bo.ef.partial      GTGTCTACCTCTGACATCCGCAGTGGTCACGTCGCCTCCGACTCCAAGAACGACCCCGCC       802
Bo.ef.CDS          GTGTCTACCTCTGACATCCGCAGTGGTCACGTCGCCTCCGACTCCAAGAACGACCCCGCC       960
                   ************************************************************

Bo.ef.partial      AAGGCCGCTGTGTCCTTCAGCGCCCAGGTCATTGTGCTTAACCACCCTGGTAACATCAAG       862
Bo.ef.CDS          AAGGCCGCTGTGTCCTTCAGCGCCCAGGTCATTGTGCTTAACCACCCTGGTAACATCAAG       1020
                   ************************************************************

Bo.ef.partial      GCTGGTTACACCCCCGTTGTTGACTGTCACACTGCCCACGTTTCCTGCAAGTTCGACGAG       922
Bo.ef.CDS          GCTGGTTACACCCCCGTTGTTGACTGTCACACTGCCCACGTTTCCTGCAAGTTCGACGAG       1080
                   ************************************************************

Bo.ef.partial      ATCACCTGCCGTATGGAC------------------------------------------       940
Bo.ef.CDS          ATCACCTGCCGTATGGACAAGCGTACTGGTAAATCCCTTGAGGAGAACCCCAAGAGCATC       1140
                   ******************

Bo.ef.partial      ------------------------------------------------------------       940
Bo.ef.CDS          AAGAATGGTGACGCCGCCATTGTCACCCTCAAGCCATGCAAGCCCATGGTCGTCGAATCC       1200


Bo.ef.partial      ------------------------------------------------------------       940
Bo.ef.CDS          TTCACTGAGTACGCCCCTCTTGGTCGTTTCGCCGTTCGTGACATGAAGCAGACTGTTGCT       1260


Bo.ef.partial      ------------------------------------------------------------       940
Bo.ef.CDS          GTCGGTGTCATCAAGAGCGTCGAGAAGAAGGAGCCTGGTTCATCTGCCAAGATCACCAAG       1320


Bo.ef.partial      ---------------------------  940
Bo.ef.CDS          TCCGCCCAGAAGGCCGCTAAGAAGTGA  1347
```

Appendix IV

Appendix IV.1: ***Babesia ovis*** **sequencing result of the amplicons obtained with primers pr-ef-ig1/ pr-ef-ovis-specific** (332 base pairs).

TTTTCTAGCTCTTGGTAGGNTGACCTACCAGCTGCTTGCGTCTGTCACTGGAAGTGGGGACACCCTGG

GGGTCTTCTGTCATTTGAAACCACATGTAACAGGCCCAGTAAAAAAAAGCACAAGAGTTATTTGATAC

CNGGGAAAACAAGAAGAAAGGGGTCCTTGCTTGGTACCCGAGGTTGTGTTTNTGATCAGAACCCCCAG

CTTGCCCGGGCTGTTTCAGTGCCACACGAACTTGGGAAGGAAACGGAGCAAGGGGAAAAGGAAACCTG

TAGACGAATGAAAAGGTATTCCATCTTCCTACAATCACGCCGGCCCAATGTAAGACNTCA

Appendix IV.2. Babesiidae annotated sequence producing significant alignments with the ***B. ovis*** **sequence obtained from amplification with primers PF-Ef-IG1 and PR-Ef-ovis-specific.**

Description	Maximum Score	Total Score	Percent Query Coverage	Expect Value	Maximum Percent Identity	GenBank Accession Number
Babesia divergens genome assembly 454hybrid_PBjelly, scaffold Contig0	33.7	33.7	8%	1.0	86%	LK934710.1
Babesia bovis mRNA for e2f-associated phosphoprotein, complete cds, clone: XBBk004120, strain: Texas	31.9	31.9	5%	3.7	100%	AK440679.1

Appendix V

Appendix V.1: *Babesia ovis* sequencing result of the amplicons obtained with primers PR-Ef-IG3/ PR-Ef-ovis-specific (745 base pairs).

ACGTGAACCGATTGTTTGGCACATATGCCATTCGTTCTTTGTTATAGGTGGGCGTGCTTGCGTGGAAC
ACTTTGACGTCTTTACACAACCTAGACCGTCACTTAAAATATGAATAGATATAGGTCCTTCACACTCC
CTGTATTTAGGTGGAGGTGTGCGCCGAGTTCGCGATGTTACAAACGGTTCTCTAGCAGTACCACTGCT
ACGCCAGAAGAATTGTCCCATTCTCCTAACCGGAAGGAGCAATTATTCTGGCAACTAAAGGTAGCATC
ATATGTATCTGGTATAGCCATATCTATCTACGGTACATATGTTTTTGTCAGCTCAGGTAAGGTCTACC
TATGAGTCTCCGTAAATGTGTGCAGGATTCCAGCTGGACAGATCCAAGCGAAAGGTTCTGTTACATTT
CTATGGCATGCTATATGGGAATACATTACCTAAACGCGTTCAAGCCTTACTGAATTCGCGATACAGTG
CTTGTTTAGATCAGGATGTGTTACGTTATCTCTCTGCATACTTTATTAAATTCGACTTGATAAAAGAC
AATGGGTTTACACGTTGAGACGCCGTTTTGTTTCTCGAAAATATCGGCATGGACGCCACCGATCCCTT
TGTGGCCCAGTTTATCGCAGCAGCAAGTGGTGATTCAATGGAACGCCGTATGGTCATCGGTTGCTCTC
TTCAGGAATTCGCTGAACTCATTGAGGCGCTTGTGTTAGAAGAGAAAATAAAGACAGCAAGTTAC

Appendix V.2 *Babesia ovis elongation factor 1α* 5' flanking region sequence retrieved from *B. ovis* Israeli strain draft genome (Bo_EFG 5'UTR placed at the end of the contig).

GCGTGTCTTCGGTACATCAGTGGAGACGTCCACTGTGTGTATTCTGTTGTATAGTTGTGTATAATTGC
GCTGCAAATTTGTGTATTGTGCTTGGTGTAGCTTGTTCTGGCCATACTAACCACTCGCAGATTGGCCT
TTGCTAACGTTTAACCAAA

Appendix VI

Appendix VI.1: *Babesia ovis rhoptry protein* gene and 3' end sequence obtained in the present study.

TTGTCAACTGtCTTnGCATAACGACCCAATTAGTGCTAACATTCATGTACCACTTTTGTTTa
CCATGATTCTAAATATTGCGTTAATTATTTTCACAGCTCGTTCTATTTCAGTAGGATGCCTT
CAACCAACTATGGTAGTAACACTTGACAAAGCGCATTCGTTTTAACCTTTATAATTCGGGTA
ATATGACGTTATGCAATGAAAATATTAGTCTATCGACAATTGCTGATTGAATCCTACACATT
TCACTATTTATAAACGACGACATAACATATGCTGGTAGCCTTTTATATAAGTATATTACGTC
TAAAGCAATCCGTATAGGTTAACAAGACGCAATAGAGGTAAGCAACGTTCTGAGTTCGGTTA
TTGTTAGTATTTTTTATAAAATAACAAAGAAATTATTTGTTACGTTGGAACCATAGTTGAGC
GATAACAATGTAGCCACATGGTTGTATTCGTCAACAATGCAATGGAGCTCGTTCtTTTTTTT
ATTAAAGAATAAATGGATAACGGCGATACATTCCTTGTGGTGGCTACATTTTCAAAGGTGGA
AGATAACCAATATCAGACACCGATACACCATTTCCTATAGGTGGCTTAATCATTACATTGTC
TTCTGTCGTTTGTATTTTTAGACAAACATTTCACCGACAGTGTTACCTCCGTCATGATTCTC
CATGTCAACGGTTTCGGTAGCTACATCGGCTACACTATCGTTATTCATCCTTTGGGGTGAGC
TAATGTAGCGCCAATTTACAGGGCGCTTGTGTCCCAAGTTCTCAAATGATTCTTTAATGGGG
GCACCAATCTTGTTCTCAAAGAAGTCCTTGGTAGGAGCACCTATCTTGTTCTCGAAGAAGTC
CTTGGTGCGAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTA
CCAAGCTTGATGCATAGCTTGAG

Appendix VII

Appendix VII.1: Alignment between the clone3a sequence and the expected pBbovis sequence.

M13F-pUC (-40) →

```
Clone 3a      ----------------------------------------------------------t     1
Expected      caccacacccgccgcgcttaatgcgccgctacagggcgcgtcccattcgccattcaggct   480
                                                                         *

Clone 3a      gcGCaActGTTGGGaAgGGCgAtCGGTGCnGGCCnttTttcgctaTTACgccAgcTGGCGa   61
Expected      gcgcaactgttgggaagggcgatcggtgc-gggcctcttcgctattacgccagctggcga   539
              ***************************** ** * * ************************

Clone 3a      AAngGGGATgTGCTGCAAGGCGATTAAGTTGGGTAACgncaGGGttTTcCCAGTCACGAC   121
Expected      aaggggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgac   599
              ** ********************************** ********************

Clone 3a      GTTGTAAAACGACngGcCAGTGAATTGTAATACGACTCACTATAGGGCGAATTgGGTACC   181
Expected      gttgtaaaacgac-ggccagtgaattgtaatacgactcactatagggcgaattgggtacc   658
              ************* ********************************************

                                                  HindIII
Clone 3a      GGGcccCCCcCTCGAGGTcgACGGTATCGATAAGCTTCACGTAATAAATGAGATAAATAAG   241
Expected      gggcccccccctcgaggtcgacggtatcgataAGCTTcacgtaataaatgagataaataag   718
              ************************************************************

Clone 3a      TATATGTCATGTATAAATTTGGGGTATAAntTTAATTGTAGAAGGTGGAGATAGTACGGT   301
Expected      tatatgtcatgtataaatttggggtataattttaattgtagaaggtggagatagtacggt   778
              ***************************** ****************************

Clone 3a      AAAAACTGAGATGATATATAATATATGATGAATATATAAACTTGACAGGTCAAGATTACT   361
Expected      aaaaactgagatgatatataatatatgatgaatatataaacttgacaggtcaagattact   838
              ************************************************************

Clone 3a      TGATCAGATTTAATTAGTTGTGATAAAGCGGAAATTCCCATATATGTCATCCCGTTAAAG   421
Expected      tggtcagatttaattagttgtgataaagcggaaattcccatatatgtcatcccgttaaag   898
              ** ********************************************************

Clone 3a      GTATTAATAGCATTTTAAGAATATATTTCACAAATATGATATACCATACATCTGATAGAA   481
Expected      gtattaatagcattttaagaatatatttcacaaatatgatataccatacatctgatagaa   958
              ************************************************************

Clone 3a      TTATGCATCGATATTCGTCGTTATAGGCTTAGTTGCGCACAACGAAAAGGTATGTGTATA   541
Expected      ttatgcatcgatattcgtcgttataggcttagttgcgcacaacgaaaaggtatgtgtata   1018
              ************************************************************

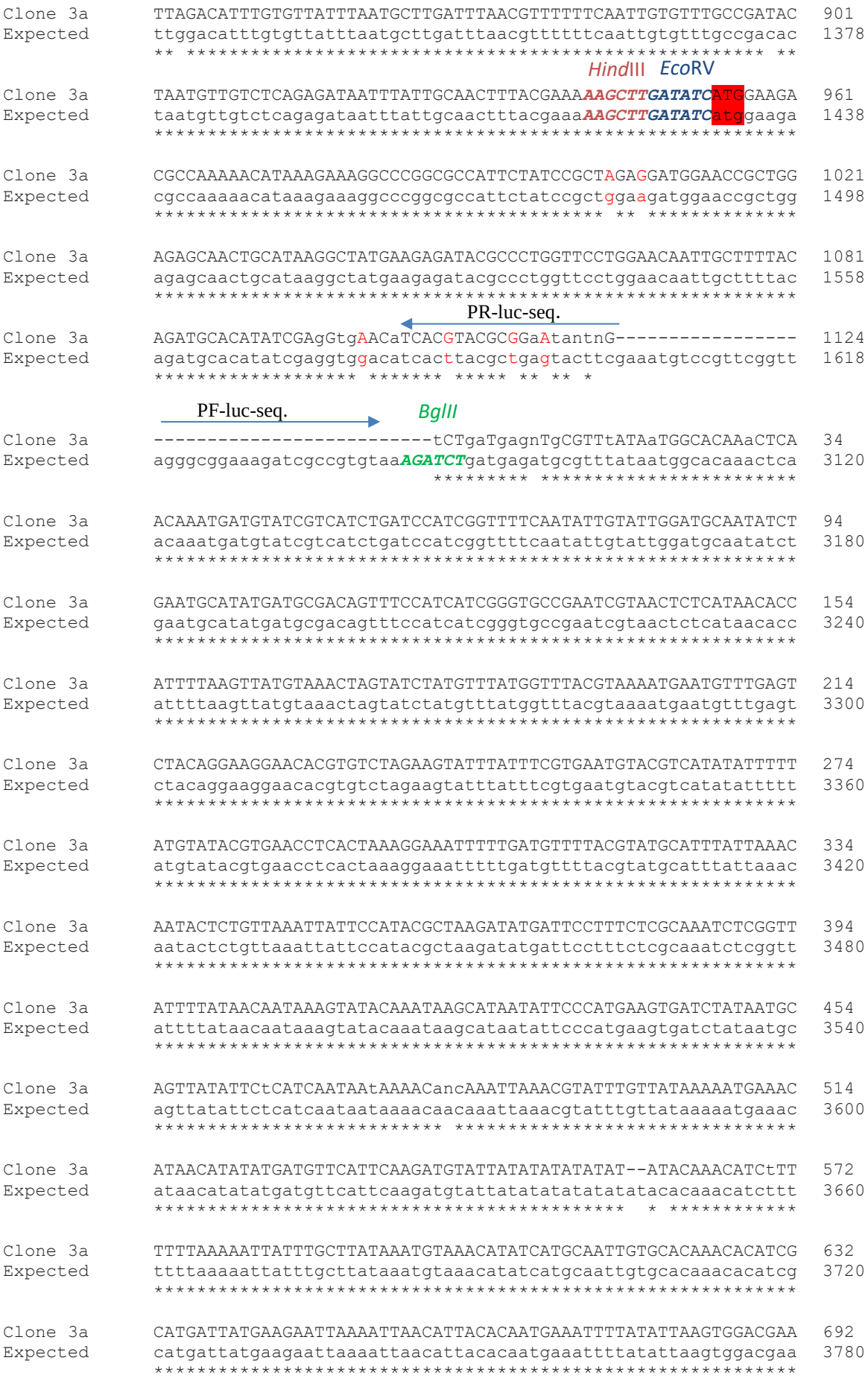
Clone 3a      TACTTAATTATATTTCAATCAAACGATGGGTGACAAATGTGTGGCGTGGAGCAAAGCAAA   601
Expected      tacttaatgatatttcaatcaaacgatgggtgacaaatgtgtggcgtggagcaaagcaaa   1078
              ******** ***************************************************

Clone 3a      ACAGCACCACAACCTTTACAAAGGGATCTTCATTAGTCCTTTGCAGTGTCTTTATAACTT   661
Expected      acagcaccacaacctttacaaagggatcttcattagtcctttgcagtgtctttataactt   1138
              ************************************************************

Clone 3a      AATAAAGTAATTCCACGCAAGAATAGTGATTATATCGCCAACAACGCACTTTTGTGATGT   721
Expected      aataaagtaattccacgcaagaatagtgattatatcgccaacaacgcacttttgtgatgt   1198
              ************************************************************

Clone 3a      AAGTCAGGCCCCTGTCATTTTGCTTTGTGGGTGGTGTTATTTCATGAAGCAGGCGACTAC   781
Expected      aagtcaggcccctgtcattttgctttgtgggtggtgttatttcatgaagcaggcgactac   1258
              ************************************************************

Clone 3a      TTCTTGTCGAGCTGCTGTGTGTATATTCTACTTTTGAGGCTATTATATACACTGATGTGT   841
Expected      ttcttgtcgagctgctgtgtgtatatcctacttttgaggctattatatacactgatgtgt   1318
              ************************** *********************************
```


Clone 3a TTAGACATTTGTGTTATTTAATGCTTGATTTAACGTTTTTTCAATTGTGTTTGCCGATAC 901
Expected ttggacatttgtgttatttaatgcttgatttaacgtttttttcaattgtgtttgccgacac 1378
HindIII EcoRV
Clone 3a TAATGTTGTCTCAGAGATAATTTATTGCAACTTTACGAAAAAGCTTGATATCATGGAAGA 961
Expected taatgttgtctcagagataatttattgcaactttacgaaaAAGCTTGATATCatggaaga 1438
Clone 3a CGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTAGAGGATGGAACCGCTGG 1021
Expected cgccaaaaacataaagaaaggcccggcgccattctatccgctggaagatggaaccgctgg 1498
Clone 3a AGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTAC 1081
Expected agagcaactgcataaggctatgaagagatacgccctggttcctggaacaattgcttttac 1558
PR-luc-seq.
Clone 3a AGATGCACATATCGAgGtgAACaTCACGTACGCGGaAtantnG---------- 1124
Expected agatgcacatatcgaggtggacatcacttacgctgagtacttcgaaatgtccgttcggtt 1618
PF-luc-seq.
BglII
Clone 3a ----------tCTgaTgagnTgCGTTtATAaTGGCACAAaCTCA 34
Expected agggcggaaagatcgccgtgtaaAGATCTgatgagatgcgtttataatggcacaaactca 3120
Clone 3a ACAAATGATGTATCGTCATCTGATCCATCGGTTTTCAATATTGTATTGGATGCAATATCT 94
Expected acaaatgatgtatcgtcatctgatccatcggttttcaatattgtattggatgcaatatct 3180
Clone 3a GAATGCATATGATGCGACAGTTTCCATCATCGGGTGCCGAATCGTAACTCTCATAACACC 154
Expected gaatgcatatgatgcgacagtttccatcatcgggtgccgaatcgtaactctcataacacc 3240
Clone 3a ATTTTAAGTTATGTAAACTAGTATCTATGTTTATGGTTTACGTAAAATGAATGTTTGAGT 214
Expected attttaagttatgtaaactagtatctatgtttatggtttacgtaaaatgaatgtttgagt 3300
Clone 3a CTACAGGAAGGAACACGTGTCTAGAAGTATTTATTTCGTGAATGTACGTCATATATTTTT 274
Expected ctacaggaaggaacacgtgtctagaagtatttatttcgtgaatgtacgtcatatattttt 3360
Clone 3a ATGTATACGTGAACCTCACTAAAGGAAATTTTTGATGTTTTACGTATGCATTTATTAAAC 334
Expected atgtatacgtgaacctcactaaaggaaatttttgatgttttacgtatgcatttattaaac 3420
Clone 3a AATACTCTGTTAAATTATTCCATACGCTAAGATATGATTCCTTTCTCGCAAATCTCGGTT 394
Expected aatactctgttaaattattccatacgctaagatatgattcctttctcgcaaatctcggtt 3480
Clone 3a ATTTTATAACAATAAAGTATACAAATAAGCATAATATTCCCATGAAGTGATCTATAATGC 454
Expected attttataacaataaagtatacaaataagcataatattcccatgaagtgatctataatgc 3540
Clone 3a AGTTATATTCtCATCAATAAtAAAACancAAATTAAACGTATTTGTTATAAAAATGAAAC 514
Expected agttatattctcatcaataataaaacaacaaattaaacgtatttgttataaaaatgaaac 3600
Clone 3a ATAACATATATGATGTTCATTCAAGATGTATTATATATATATAT--ATACAAACATCtTT 572
Expected ataacatatatgatgttcattcaagatgtattatatatatatatatacacaaacatcttt 3660
Clone 3a TTTTAAAAATTATTTGCTTATAAATGTAAACATATCATGCAATTGTGCACAAACACATCG 632
Expected ttttaaaaattatttgcttataaatgtaaacatatcatgcaattgtgcacaaacacatcg 3720
Clone 3a CATGATTATGAAGAATTAAAATTAACATTACACAATGAAATTTTATATTAAGTGGACGAA 692
Expected catgattatgaagaattaaaattaacattacacaatgaaattttatattaagtggacgaa 3780

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Clone 3a      TTAGGTTTATGTTTACATATAAGGTGGTGATATGTTTGGTCTTATGATGTTGTTATACAC  752
Expected      ttaggtttatgtttacatataaggtggtgatatgtttggtcttatgatgttgttatacac  3840
              ************************************************************

Clone 3a      ATTCATGGCACGACATTTTGATAGTTTAGTAATGAAGTCTAGTTAATGTTGCCAATGCTA  812
Expected      attcatggcacgacattttgatagtttagtaatgaagtctagttaatgttgccaatgcta  3900
              ************************************************************

 Clone 3a     GCCAATTAAAATAGGCTAAATTATATTTTTATGAATAATTTCTTTTTTTtCCAATCCACG  872
Expected      gccaattaaaatataggctaaattatatttttatgaataatttctttttttccaatccacg  3960
              ************************************************************

Clone 3a      ATGGCAGACTTGGCAACAGCAATTGCTGAATTTGATTCTCTTAACGAATTTTCAAAACCT  932
Expected      atggcagacttggcaacagcaattgctgaatttgattctcttaacgaattttcaaaacct  4020
              ************************************************************

Clone 3a      GATGCTAAATTATTATCCACAATACTACTATCGGATGGCGTAAAATTATCACAACAACTT  992
Expected      gatgctaaattattatccacaatactactatcggatggcgtaaaattatcacaacaactt  4080
              ************************************************************

Clone 3a      AAGGCGCTTTACTTCTGTCGTGATTTATCTTCTAGTGAATGTGCTACTATCCTTAAAAAG  1052
Expected      aaggcgctttacttctgtcgtgatttatcttctagtgaatgtgctactatccttaaaaag  4140
              ************************************************************

Clone 3a      GCACTTGAAGTACACTACGATACATTCTTGAGGTAGGTAATAGCACGTAATGTTATTATG  1112
Expected      gcacttgaagtacactacgatacattcttgaggtaggtaatagcacgtaatgttattatg  4200
              ************************************************************

Clone 3a      TTACCTTATTCATTCTTCATCTTCTTATAGACATGAAATTGCTTATGTAATTGGACAAGC  1172
Expected      ttaccttattcattcttcatcttcttatagacatgaaattgcttatgtaattggacaagc  4260
              ************************************************************

Clone 3a      AGAATGCGAGGAAGCAGCTGACGTATTGGTTCGCCTTTTGGAGGATACTAATGAAGACCC  1232
Expected      agaatgcgaggaagcagctgacgtattggttcgccttttggaggatactaatgaagaccc  4320
              ************************************************************
                                                          BamHI
Clone 3a      TATGGTCAGACATGAGGTATGTCGTTTAACTCTTTGACATATCGTTCGTAGGGGATCCAC  1292
Expected      tatggtcagacatgaggtatgtcgtttaactctttgacatatcgttcgtagggggatccac  4380
              ************************************************************
                                          M13F-pUC (-40)
Clone 3a      TAGTTCTAGAGCGGCCGCCACCGCGGtggAGCTCCAGCTTTTgttCCCttTAg-------  1345
Expected      tagttctagagcggccgccaccgcggtggagctccagcttttgttccctttagtgagggt  4440
              ************************************************
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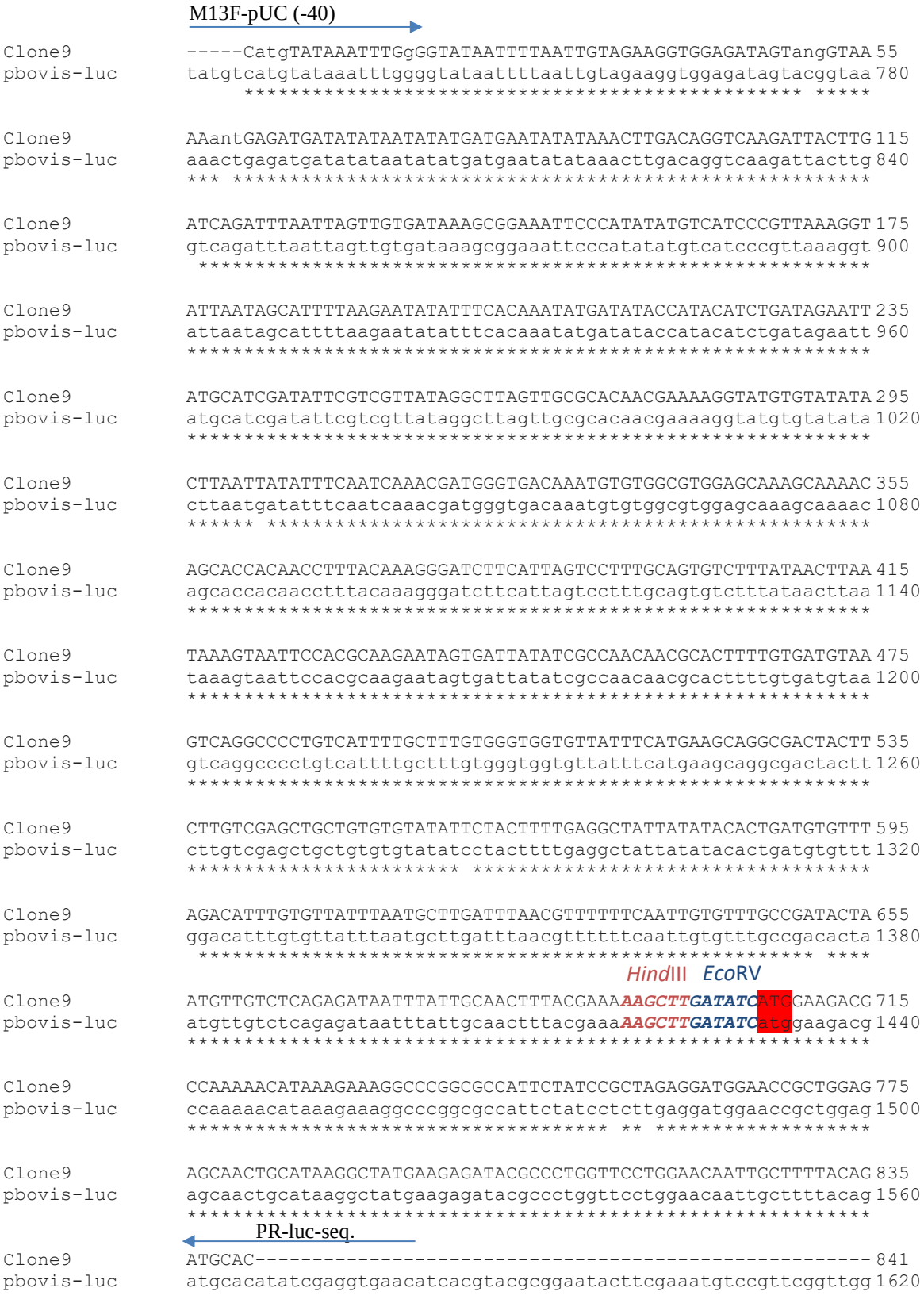
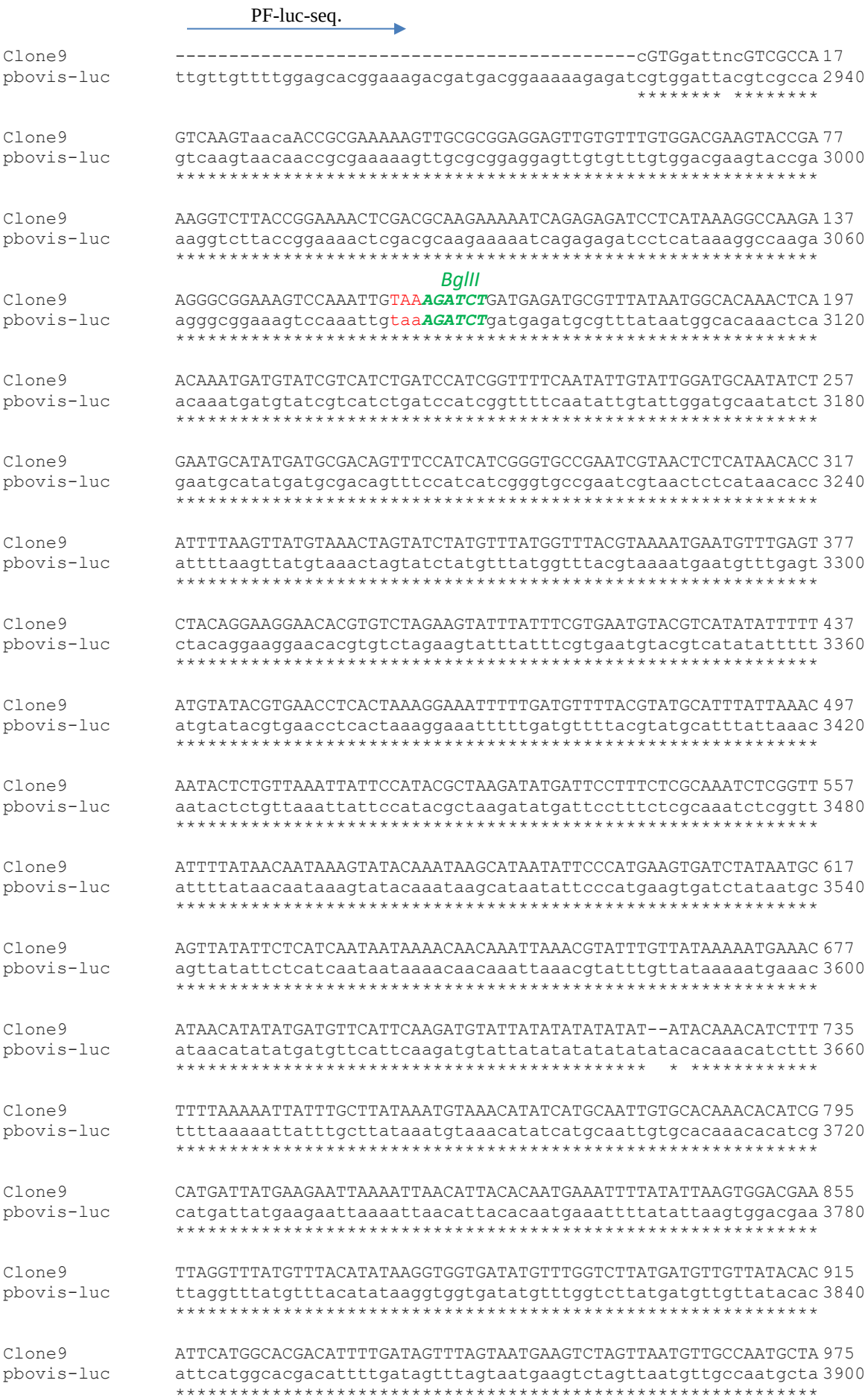
Appendix VIII

Appendix VIII.1: Alignment between the clone9 sequence and pBbovis-luc sequence.

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BglII
Clone9 ----------cGTGgattncGTCGCCA 17
pbovis-luc ttgttgttttggagcacggaaagacgatgacggaaaaagagatcgtggattacgtcgcca 2940
******** ********
Clone9 GTCAAGTaacaACCGCGAAAAAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGA 77
pbovis-luc gtcaagtaacaaccgcgaaaaagttgcgcggaggagttgtgtttgtggacgaagtaccga 3000
Clone9 AAGGTCTTACCGGAAAACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGA 137
pbovis-luc aaggtcttaccggaaaactcgacgcaagaaaaatcagagagatcctcataaaggccaaga 3060
Clone9 AGGGCGGAAAGTCCAAATTGTAAAGATCTGATGAGATGCGTTTATAATGGCACAAACTCA 197
pbovis-luc agggcggaaagtccaaattgtaaAGATCTgatgagatgcgtttataatggcacaaactca 3120
Clone9 ACAAATGATGTATCGTCATCTGATCCATCGGTTTTCAATATTGTATTGGATGCAATATCT 257
pbovis-luc acaaatgatgtatcgtcatctgatccatcggttttcaatattgtattggatgcaatatct 3180
Clone9 GAATGCATATGATGCGACAGTTTCCATCATCGGGTGCCGAATCGTAACTCTCATAACACC 317
pbovis-luc gaatgcatatgatgcgacagtttccatcatcgggtgccgaatcgtaactctcataacacc 3240
Clone9 ATTTTAAGTTATGTAAACTAGTATCTATGTTTATGGTTTACGTAAAATGAATGTTTGAGT 377
pbovis-luc attttaagttatgtaaactagtatctatgtttatggtttacgtaaaatgaatgtttgagt 3300
Clone9 CTACAGGAAGGAACACGTGTCTAGAAGTATTTATTTCGTGAATGTACGTCATATATTTTT 437
pbovis-luc ctacaggaaggaacacgtgtctagaagtatttatttcgtgaatgtacgtcatatatttttt 3360
Clone9 ATGTATACGTGAACCTCACTAAAGGAAATTTTTGATGTTTTACGTATGCATTTATTAAAC 497
pbovis-luc atgtatacgtgaacctcactaaaggaaatttttgatgttttacgtatgcatttattaaac 3420
Clone9 AATACTCTGTTAAATTATTCCATACGCTAAGATATGATTCCTTTCTCGCAAATCTCGGTT 557
pbovis-luc aatactctgttaaattattccatacgctaagatatgattcctttctcgcaaatctcggtt 3480
Clone9 ATTTTATAACAATAAAGTATACAAATAAGCATAATATTCCCATGAAGTGATCTATAATGC 617
pbovis-luc attttataacaataaagtatacaaataagcataatattcccatgaagtgatctataatgc 3540
Clone9 AGTTATATTCTCATCAATAATAAAACAACAAATTAAACGTATTTGTTATAAAAATGAAAC 677
pbovis-luc agttatattctcatcaataataaaacaacaaattaaacgtatttgttataaaaatgaaac 3600
Clone9 ATAACATATATGATGTTCATTCAAGATGTATTATATATATATAT--ATACAAACATCTTT 735
pbovis-luc ataacatatatgatgttcattcaagatgtattatatatatatatatacacaaacatcttt 3660
Clone9 TTTTAAAAATTATTTGCTTATAAATGTAAACATATCATGCAATTGTGCACAAACACATCG 795
pbovis-luc ttttaaaaattatttgcttataaatgtaaacatatcatgcaattgtgcacaaacacatcg 3720
Clone9 CATGATTATGAAGAATTAAAATTAACATTACACAATGAAATTTTATATTAAGTGGACGAA 855
pbovis-luc catgattatgaagaattaaaattaacattacacaatgaaattttatattaagtggacgaa 3780
Clone9 TTAGGTTTATGTTTACATATAAGGTGGTGATATGTTTGGTCTTATGATGTTGTTATACAC 915
pbovis-luc ttaggtttatgtttacatataaggtggtgatatgtttggtcttatgatgttgttatacac 3840
Clone9 ATTCATGGCACGACATTTTGATAGTTTAGTAATGAAGTCTAGTTAATGTTGCCAATGCTA 975
pbovis-luc attcatggcacgacattttgatagtttagtaatgaagtctagttaatgttgccaatgcta 3900


Clone9 GCCAATTAAAATAGGCTAAATTATATTTTTATGAATAATTTCTTTTTTTtCCAATCCACG 1035
pbovis-luc gccaattaaaataggctaaattatatttttatgaataatttctttttttccaatccacg 3960
Clone9 ATGGCAGACTTGGCAACAGCAATTGCTGAATTTGATTCTCTTAACGAATTTTCAAAACCT 1095
pbovis-luc atggcagacttggcaacagcaattgctgaatttgattctcttaacgaattttcaaaacct 4020
Clone9 GATGCTAAATTATTATCCACAATACTACTATCGGATGGCGTAAAATTATCACAACAACTT 1155
pbovis-luc gatgctaaattattatccacaatactactatcggatggcgtaaaattatcacaacaactt 4080
Clone9 AAGGCGCTTTACTTCTGTCGTGATTTATCTTCTAGTGAATGTGCTACTATCCTTAAAAAG 1215
pbovis-luc aaggcgctttacttctgtcgtgatttatcttctagtgaatgtgctactatccttaaaaag 4140
Clone9 GCACTTGAAGTACACTACGATACATTCTTGAGGTAGGTAATAGCACGTAATGTTATTATG 1275
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Clone9 AGAATGCGAGGAAGCAGCTGACGTATTGGTTCGCCTTTTGGAGGATACTAATGAAGACCC 1395
pbovis-luc agaatgcgaggaagcagctgacgtattggttcgcctttggaggatactaatgaagaccc 4320
BamHI
Clone9 TATGGTCAGACATGAGGTATGTCGTTTAACTCTTTGACATATCGTTCGTAGGGGATCCCAC 1455
pbovis-luc tatggtcagacatgaggtatgtcgtttaactctttgacatatcgttcgtaggggatccac 4380
M13F-pUC (-40)
Clone9 TAGTTCTAGAGCGGCCGCCACCGCGgtggAGCTCCAGCTTTTgttCCCttTAg------- 1508
pbovis-luc tagttctagagcggccgccaccgcggtggagctccagcttttgttcccttagtgagggt 4440

Appendix IX

Appendix VIII.1: Alignment between the clone3 sequence and pBovata-luc sequence.

M13F-pUC (-40)

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clone3          -----------------GCCGGGATGTATACGACTCACTATAGGGCGAATTGGGTACCGG 43
povata-luc      TTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGTACCGG 660
                                 *         **********************************

clone3          GCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTCACTCATTTAGATTGCGACATGTTAC 103
povata-luc      GCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTCACTCATTTAGATTGCGACATGTTAC 720
                ************************************************************

clone3          AAGTAATGTGGCGTTCAGTATGCTTTCTGGGATGCCTAAAAGACGATGTGCGGCACGCCT 163
povata-luc      AAGTAATGTGGCGTTCAGTATGCTTTCTGGGATGCCTAAAAGACGATGTGCGGCACGCCT 780
                ************************************************************

clone3          CAGCGTAATCCCACGTTAGACATACAAACGTTGTGATGAAGTTGCAATGTTAGTTATGTG 223
povata-luc      CAGCGTAATCCCACGTTAGACATACAAACGTTGTGATGAAGTTGCAATGTTAGTTATGTG 840
                ************************************************************

clone3          TATTAAGCGTATATAAGTAACTTCGAGTTATCACAGCATTCTGCCGCGAAATTGCTCGCG 283
povata-luc      TATTAAGCGTATATAAGTAACTTCGAGTTATCACAGCATTCTGCCGCGAAATTGCTCGCG 900
                ************************************************************

clone3          GAATGTGATTGGCGTGTCACGAAAAATTTTACCACAACATTGCCGGCTATTATATGCACA 343
povata-luc      GAATGTGATTGGCGTGTCACGAAAAATTTTACCACAACATTGCCGGCTATTATATGCACA 960
                ************************************************************

clone3          GAACGTTCATACACTTTTGTGTAAAGCGATGCATCATAAGTAAAAGTGTGTGGTCTTGCT 403
povata-luc      GAACGTTCATACACTTTTGTGTAAAGCGATGCATCATAAGTAAAAGTGTGTGGTCTTGCT 1020
                ************************************************************

clone3          GTGCCTGTTCGGTGAACGGCGCGTGAGATGGTCTAATGCGATAATATAATACTGCTTTTA 463
povata-luc      GTGCCTGTTCGGTGAACGGCGCGTGAGATGGTCTAATGCGATAATATAATACTGCTTTTA 1080
                ************************************************************

clone3          CACATTAAACAAATGACAATGGTACTGCTAAAAAAGGTGTTCAATGAACATCCTGAAGAG 523
povata-luc      CACATTAAACAAATGACAATGGTACTGCTAAAAAAGGTGTTCAATGAACATCCTGAAGAG 1140
                ************************************************************

clone3          TAATTCGGTTTCATCCGCTGTTACCACTATATACCGGTTTACGGCGATATTACCCCACTG 583
povata-luc      TAATTCGGTTTCATCCGCTGTTACCACTATATACCGGTTTACGGCGATATTACCCCACTG 1200
                ************************************************************

clone3          CATCCTCACGCCGTATATTGAACGGAAATAAGTCACTACCATTCACTCGCGCTCTGCCGG 643
povata-luc      CATCCTCACGCCGTATATTGAACGGAAATAAGTCACTACCATTCACTCGCGCTCTGCCGG 1260
                ************************************************************

clone3          TACCAGTTTTATAAAACGCAGGTACAATCTAATTGATTTATATTGTAAGTTTCGTAGCAT 703
povata-luc      TACCAGTTTTATAAAACGCAGGTACAATCTAATTGATTTATATTGTAAGTTTCGTAGCAT 1320
                ************************************************************

clone3          CGCCGTTTTGTGTTACGTGTGGGTGTGATGGTTTAGACGGCGCGTCCGTGGACGGCCGTT 763
povata-luc      CGCCGTTTTGTGTTACGTGTGGGTGTGATGGTTTAGACGGCGCGTCCGTGGACGGCCGTT 1380
                ************************************************************

clone3          TTGCGTGTGTATGGTAGGCCACGGTTTCGCAACGATGCTGCGTCGCCTTGGTTGGTGATT 823
povata-luc      TTGCGTGTGTATGGTAGGCCACGGTTTCGCAACGATGCTGCGTCGCCTTGGTTGGTGATT 1440
                ************************************************************

clone3          CGTGGTGTATGGGCCATTTTGTGGTATTGTCGTGCGTCACACTCCATTAGACCCGTAGCT 883
povata-luc      CGTGGTGTATGGGCCATTTTGTGGTATTGTCGTGCGTCACACTCCATTAGACCCGTAGCT 1500
                ************************************************************
```

*Hind*III *Eco*RV

```
clone3          AACTGTGCGCAGGTTTGCTATCGTTAAACCTTAAACAAGAAGCTTGATATCATGGAAGAC 943
povata-luc      AACTGTGCGCAGGTTTGCTATCGTTAAACCTTAAACAAGAAGCTTGATATCATGGAAGAC 1560
                ************************************************************

clone3          GCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTAGAGGATGGAACCGCTGGA 1003
povata-luc      GCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCTCTTGAGGATGGAACCGCTGGA 1620
                ************************************ ** ******************

clone3          GAGCAACTGCATAAGGCTATGAAGAGATACCCCCTGGTTCCTGGAACAATTGCTTTTACA 1063
povata-luc      GAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACA 1680
                ***************************** ****************************

clone3          GATGCACATATCGAGGTGAAC---------------------------------------- 1084
povata-luc      GATGCACATATCGAGGTGAACATCACGTACGCGGAATACTTCGAAATGTCCGTTCGGTTG 1740
                ********************
```

Appendix X

Appendix X.1: Representative pictures of *Babesia ovis* with and without blasticidin-S at 72h. Black arrows indicate *B. ovis* with a regular phenotype. Intraerythrocytic parasites were observed under a 400x original magnification of a Motic BA210 LED trinocular compound microscope.